University of Alberta

Response of Bacteriocin Resistant *Listeria monocytogenes* Strains to Preservation Factors

by

Juan Ignacio Aguilar Guerrero

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Spring, 2004

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ISBN: 0-612-96438-8

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Abstract

The relationship between the acquisition of bacteriocin resistance in strains of *Listeria monocytogenes* was studied and the resistance of these strains to preservation factors such as temperature, acidity and high salt concentration. Resistant strains were obtained by exposure of test organisms to three different bacteriocins. Bacteriocin-resistant strains that were stable for 100 generations were obtained. Leucocin-resistant strains of *L. monocytogenes* ATCC 15313 were more thermo-, salt- and acid-tolerant ($p<0.05$) than the wild-type strain. Acid tolerance of brochocin-C resistant strains of *L. monocytogenes* Scott A and the brochocin-C/nisin-resistant strains was significantly ($p<0.05$) greater than the wild-type strains; whereas, acid tolerance of leucocin A-resistant strains was not significantly different than the wild-type (ATCC 15313) strain. The study demonstrated that acquisition of resistance to bacteriocins in strains of *L. monocytogenes* could alter the resistance to other preservation factors.

Acknowledgements

I would like to express my sincere gratitude to my supervisors, Dr. Lynn M. McMullen and Dr. Michael E. Stiles, for their guidance, support and for sharing with me their passion for science.

I would like to extend my appreciation to MEng. Rosa Sanchez for her assistance in the preservation experiments. I also thank PhD Candidate Erick Silva for his help on the statistical analysis.

I would like to thank the fellow graduate students and staff in the Food Microbiology Laboratory.

I would like to thank the Consejo Nacional de Ciencia y Tecnología (CONACYT), Dr. Lynn McMullen and CanBiocin Inc. for the financial support during my studies at the University of Alberta.

I would like to express my gratitude to Nancy and Brian Penny, Betty and Bob Volker, Ethel Seutter, , Denise Delong and Familia Silva-Prado for being the surrogate kinfolk who welcomed me and my family to their homes and lives.

Finally, I would like to thank my wife, Mireya Merlin for put all her bets on this endeavor and for her support and encourage to pursue this degree.

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List of Abbreviations

ANOVA	Analysis of variance
APT	All Purpose Tween
ASP	Acid Shock Protein
ATCC	American Type Culture Collection
ATR	Acid Tolerance Response
AU	Arbitrary units
bp	Base pair
bro^r	Brochocin-C resistant strain
BSH	bile salt hydrolase
CFU	Colony forming units
Da	Dalton
DNA	Deoxyribonucleic acid
DnaJ	Heat shock protein 40
DnaK	Heat shock protein 70
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
GABA	γ-aminobutyrate
GAD	Glutamate Decarboxylase acid resistance system
Gly	Glycine
GSP	general stress proteins
GroEL	Heat shock protein 60
h	Hour
HK	Histidine kinase
HSP	Heat Shock Proteins
IU	International Unit
Kb	Kilobase
LAB	Lactic Acid Bacteria
leu^r	Leucocin A resistant strain

min	Minute
ml	Milliliter
MRS	de Man, Rogosa and Shape
PGs	phosphatidylglycerol
PMF	proton motive force
PTS	phosphotransferase system
RAPD	Random Amplification of polymorphic DNA
RNA	Ribonucleic acid
rpm	Revolutions Per Minute
RpoS	Sigmas factor
RR	Response regulator
Sec	Secretory
TE	Tris and ethylenediaminetetraacetic acid buffer
TFA	Trifluoroacetic acid
TSBYE	Trypticase Soy Broth supplemented with 0.6% yeast extract
UAL	University of Alberta Lactic Acid Bacteria collection
USA	Unites States of America
σ^B	Sigma B

1 Introduction

Listeria monocytogenes is a Gram-positive pathogen that can be foodborne and is frequently found in the food processing environment. It is commonly found in the intestines of humans and animals, in raw milk, soil, on leafy vegetables, and in food processing environments (Ryser and Marth 1999), and it can survive for long periods of time in foods and in the environment, particularly at refrigeration or frozen storage temperatures. Among animals, *Listeria* has a long history in veterinary medicine as a cause of abortion and encephalitis and eventually death, especially in sheep. It is commonly associated with feeding silage. *L. monocytogenes* causes illness by penetrating the lining of the gastrointestinal tract and then infecting normally sterile sites within the body. The likelihood that *L. monocytogenes* will invade the intestinal tissue depends upon a number of factors, including the number of organisms consumed, host susceptibility and virulence of the specific bacterial strain ingested.

L. monocytogenes infections are primarily recorded in pregnant women, neonates, immunocompromised individuals and the elderly (Farber and Peterkin 1991). These vulnerable groups form a considerable proportion of the population. The incubation period before onset of the disease can be as long as ten weeks, and this causes considerable difficulty in determining the food implicated in the infection.

L. monocytogenes was first recognized as a foodborne pathogen in Germany in 1953 when twins were stillborn and the infection was linked to consumption of raw milk from a cow with listerial mastitis. However, it was not until several large, common source

outbreaks of listeriosis occurred in Canada, the United States of America (USA) and Europe during the 1980s that the significance of foods as the primary route of transmission for human exposure to *L. monocytogenes* was recognized (Bille 1990; Broome *et al.* 1990). It is generally considered that most cases of human listeriosis involve foodborne transmission. Recognition of the hazard caused by this organism has led to a tightening of the control of hygiene during the production of foods. Measures have been taken in many countries to reduce the previously widespread occurrence of *L. monocytogenes* in processing plants.

In 1981 in Nova Scotia, Canada, coleslaw consumption was associated with listeriosis involving 41 cases (Schlech *et al.* 1983). In 1985 Mexican-style cheese contaminated with *L. monocytogenes* was directly linked to an outbreak that caused at least 142 cases of listeriosis in the USA including 48 deaths (Linnan *et al.* 1988). Another outbreak due to consumption of soft cheese occurred in Switzerland with 122 cases of listeriosis and 31 deaths (Bille 1990). A multistate outbreak of listeriosis occurred in USA between October 1998 and February 1999 in which approximately 100 people were diagnosed with listerial infection and 21 died. Hot dogs were implicated as the food involved in the outbreak (Centers for Disease Control and Prevention 1999). As a result, a major food-processing company voluntarily recalled the product that resulted in what has been estimated to be the largest food recall in USA. As a result of these outbreaks, regulatory agencies in the USA adopted a "zero tolerance" (no detectable level permitted) policy for *L. monocytogenes* in ready-to-eat (RTE) foods. This policy was based on minimal data about the prevalence and control of this foodborne pathogen. Since then, more research

has revealed more knowledge of the source, the food carrier, and the populations affected by this pathogen. With this knowledge, food producers have endeavoured to improve sanitation and to implement food safety programs such as Hazard Analysis and Critical Control Points (HACCP). As a further consequence, the food industry has increased the intensity of their sampling programs for *Listeria* on RTE meat and poultry products. Numerous food recalls have resulted when manufacturers find *Listeria* in RTE foods.

The use of different physical and chemical methods to control *Listeria* in foods has been reported, including heating, freezing, drying, exposure to acids and to disinfectants, and to high osmotic pressure (Farber and Peterkin 1991; Linton *et al.* 1992; Lou and Yousef 1997; Robinson *et al.* 1997). A new approach using lactic acid bacteria (LAB) as a competitive microflora in foods has also been studied, in some cases these strain produced biological compounds such as bacteriocins, which were responsible for the inhibition. Bacteriocins are small antimicrobial peptides (Jack *et al.* 1995) that are produced by many strains of LAB including members of the genera: *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Enterococcus*, and *Pediococcus* (Nes *et al.* 1996)

A major subgroup of bacteriocins is Class IIa, not only because of the large number that have been characterized, but also because of their inhibitory activity against the foodborne pathogen *L. monocytogenes*. Class IIa bacteriocins have the potential for use as antimicrobial agents in food preservation (Eijsink *et al.* 1998).

Sublethal heating that induces heat shock conditions increases the thermotolerance of *L. monocytogenes* (Stephens *et al.* 1994). Bacteriocin resistance of undesirable bacterial species that are normally bacteriocin-sensitive is another concern in the effort to control *L. monocytogenes*. Studies on bacteriocin resistance have dealt with acquired resistance, which is obtained through successive contact of a sensitive strain with a bacteriocin (Mazzotta and Montville 1997; Rekhif *et al.* 1994). Changes in the bacteriocin tolerance of a foodborne pathogen such as of *L. monocytogenes* could result in inadequate inactivation of the pathogen and a phenomenon known as "stress hardening." This refers to the increased resistance to lethal factors after adaptation to environmental stresses. This could neutralize the effectiveness of food preservation hurdles and compromise food safety (Lou and Yousef 1997).

It has been demonstrated that both heat shock (Farber and Brown 1990; Linton *et al.* 1990) and osmotic shock (Jørgensen *et al.* 1995) increase the thermotolerance of *L. monocytogenes*, but it is not clear what effect, if any, the acquisition of bacteriocin-resistance has on the response of *L. monocytogenes* to other hurdles used for food preservation.

The objective of the research was to evaluate whether resistance of *L. monocytogenes* to the bacteriocins nisin, brochocin-C and leucocin A leads to increased resistance to heat, low pH and high salt concentration relative to the wild-type cells.

2 Literature review

2.1 Bacteriocins

Bacteriocins are antibacterial peptides or proteins that are produced by many bacteria that are included in the general grouping of organisms that are referred to as Lactic Acid Bacteria (LAB). These bacteriocins are ribosomally synthesized and generally consist of 20 to 60 amino acids. In most cases bacteriocins are produced as an inactive pre-peptide that is activated when the leader sequence is cleaved during secretion of the bacteriocin from the cell by a dedicated two-protein export system. In most cases no further post-translational change occurs in the peptide (nonlantibiotic) but some bacteriocins undergo quite extensive post-translational modification to produce the active compound (lantibiotics). Almost all bacteriocins have a positive charge at neutral pH and they usually contain hydrophobic and/or amphiphilic sequences.

Bacteriocins generally act on susceptible cells by creating pores in the cell membrane through electrostatic interactions between the positively charged peptide and anionic lipids in the bacterial cell membrane causing dissipation of the proton motive force, ATP depletion and leakage of nutrients and metabolites and/or through inhibition of cell wall synthesis (Abee *et al.* 1995). Bacteriocins vary in specificity from a narrow spectrum of activity (e. g., lactococcins that only inhibit other strains of lactococci) to those that have a relatively broad range of activity against other Gram-positive bacteria, such as the lantibiotic nisin (Jack *et al.* 1995) and the nonlantibiotic two-component bacteriocin Brochocin-C (McCormick *et al.* 1998). These proteinaceous inhibitors have attracted

intensive research interest over the last three decades, resulting in the discovery and characterization of many different types of bacteriocins from LAB.

There have been several attempts to classify LAB bacteriocins (Klaenhammer 1993; Nes *et al.* 1996; Nes and Holo 2000; McAuliffe *et al.* 2001; McAuliffe *et al.* 2001; van Belkum and Stiles 2000). According to Klaenhammer and Nes bacteriocins of Gram-positive bacteria can be classified in four distinct classes (Klaenhammer 1993; Nes *et al.* 1996).

Table 1. Classification of bacteriocins adapted from Klaenhammer (1993) and Nes *et al.* (1996)

Class	Subclass	Features	Examples of bacteriocins
I	Ia	Lantibiotic, small peptides containing lanthionine and β-methyl lanthionine	Nisin
	Ib	Globular peptides with no net charge or a net negative charge	Mersacidin
II	IIa	Small heat-stable peptides with antilisterial effectiviness	Pediocin PA-1, leucocin A, carnobacteriocins, etc.
	IIb	Two peptides bacteriocins	Brochocin-C, lactococcin G, lactacin F
III		Large and heat-labile molecules	Helveticin J, lacticins A and B

2.1.1 Class I: Lantibiotics

Lantibiotics are small membrane-active peptides that undergo extensive post-translational modification to produce unusual amino acids in the mature peptides. The lantibiotics

have been found in LAB and in other Gram-positive bacteria, such as staphylococci (Eijsink *et al.* 2002). These peptides form unstable pores in bacterial cell membranes. Lantibiotics are divided into two subgroups, A and B, based on structural features and their mode of killing (Jung and Sahl 1991). Type A lantibiotics kill sensitive cells by depolarizing the cytoplasmic membrane (Hechard *et al.* 2002). The best-known Type A lantibiotic is nisin. It has a broad antimicrobial spectrum and it is currently approved for use as food preservative in over 50 countries (Delves-Broughton *et al.* 1996) as well as by the Food and Agriculture Organization/World Health Organization and the European Union. The Type B lantibiotics have a globular secondary structure, they have either no net charge or a net negative charge (Altena *et al.* 2000) and they interfere with enzymes in the cell cytoplasm causing enzyme inhibition.

Nisin is produced by certain strains of *Lactococcus lactis* subsp. *lactis*. It was first described by Rogers *et al.* in 1928 as a Lancefield group N *Streptococcus* inhibitory substance. Nisin is produced as a precursor peptide that undergoes post-translational modification to produce modified amino acids such as 2, 3-didehydroalanine, D-alanine, and 2, 3-didehydrobutyrine, as well as characteristic lanthionine rings that result from thioether formation between the side chains of cysteine and serine or threonine (Gross and Morell 1971).

Nisin is inhibitory to a wide range of Gram-positive bacteria, including streptococci, staphylococci, and lactobacilli, most of the spore-forming species of *Clostridium* and *Bacillus*, and *Listeria*. Nisin is licensed for use for the preservation of certain foods. It was granted GRAS (Generally Regarded as Safe) status in the U.S. Federal Register of

April, 1988 (Federal Register 1988). This bacteriocin has been the subject of many fundamental studies on its structure and genetics (Guder *et al.* 2000; Sahl *et al.* 1995). Nisin is composed of 34 amino acids and has a pentacyclic structure with one lanthionine residue and four β-methyllanthionine residues. The nisin molecule is amphiphatic; the N-terminus of the molecule contains a relatively large number of hydrophobic residues, whereas the C-terminus is more hydrophilic (Guder *et al.* 2000).

Nisin effectively inhibits sensitive bacteria at nanomolar concentrations depending on the target bacterial strain. Nisin causes pore formation in the membranes of sensitive bacteria leading to an instant depolarisation along with of the cytoplasmic membrane, a rapid efflux of low molecular weight compounds and complete cessation of biosynthetic processes (Moll *et al.* 1996; Ruhr and Sahl 1985; Sahl *et al.* 1987; Sahl and Brandis 1982).

2.1.2 Class II bacteriocins

Class II bacteriocins are ribosomally synthesized and are amphipathic and thermo-stable. Their inhibitory spectrum is rather narrow, limited to species or strains related to the producer organisms. Consequently, class II bacteriocins are mainly active against Gram-positive bacteria, such as LAB, *Enterococcus* and *Clostridium* spp. and especially *Listeria* species. Some bacteriocins that are produced by LAB are specifically denoted as being *Listeria*-active.

Genetic analysis has shown that class II bacteriocins are synthesized as prebacteriocins with an N-terminal extension of 18 to 24 amino acid residues that are cleaved by a proteolytic processing usually after characteristic Gly-Gly residues to release the mature

bacteriocin. The leader sequence of the precursor bacteriocin is cleaved by the ABC-transporter protein that is involved in secretion of the bacteriocins.

Class II bacteriocins are divided in three subclasses, namely IIa, IIb, and IIc, on the basis of their primary structure (Klaenhammer 1993; Nes *et al.* 1996).

Class IIa bacteriocins (also called pediocin-like bacteriocins) are an abundant, highly homologous family of antimicrobial peptides that are heat-stable and are also active against the foodborne pathogen *L. monocytogenes* (Cleveland *et al.* 2001; Ennahar *et al.* 2000a). These antimicrobial peptides are characterized by the presence of YGNGV and CXXXXCXV sequence motifs in their N-terminal segments (Ennahar *et al.* 2000b; Nes *et al.* 2002) including cysteine residues that form disulfide bridges (Eijsink *et al.* 1998) as well as by their strong inhibitory effect on *Listeria* (Cintas *et al.* 1997; Kaiser and Montville 1996; Quadri *et al.* 1994). Pediocin PA-1/AcH, enterocin A and divercin V41 belong to this group in the sense that they possess an extra disulfide bond involving a second pair of cysteine residues. They show higher activity than those with only two cysteines (Eijsink *et al.* 1998; Jack *et al.* 1995). In a comparative study, Eijsink *et al.* (1998) showed that the two-disulfide-bond bacteriocins pediocin AcH and enterocin A are more efficient antibacterials than sakacin P and curvacin A, which possess a single disulfide bond, especially against strains of *Listeria*, and that they display an overall broader spectrum of activity.

Class IIa bacteriocins are thought to act primarily by permeabilizing the target membrane by the formation of pores. These pores cause leakage of ions and inorganic phosphates and subsequently dissipate the proton motive force (Chikindas *et al.* 1993; Maftah *et al.*

1993) but an initial interaction with a target molecule at the surface of a sensitive cell is likely. Mannose phosphotransferase system (PTS) permease has been cited as the proposed target molecule (Hechard *et al.* 2001; Hechard and Sahl 2002). Because of their antilisterial effectiveness, class IIa bacteriocins have significant potential as biopreservatives in a large number of foods.

Class IIb bacteriocins are poration complexes formed by oligomers of two different proteinaceous peptides (Allison *et al.* 1994; Nissen-Meyer *et al.* 1992; van Belkum *et al.* 1991). The two peptides act synergistically and have only one dedicated immunity protein whose gene is linked to those of the two structural genes for the peptides, usually in an operon structure (Nes *et al.* 1996). Antibacterial activity of the individual peptides is limited and in some cases the individual peptides are completely inactive such in brochocin-C peptides both peptides need to be present to display antibacterial activity (Garneau *et al.* 2003).

Class IIc bacteriocins contain thiol-activated bacteriocins (Klaenhammer 1993) and the excretion depends on the translocase general secretion (*sec*) pathway of the cell (Nes *et al.* 1996; van Belkum and Stiles 2000).

Class III bacteriocins consist of heat-labile bacteriocins with a molecular mass larger than 30 kDa. Most of them are produced by bacteria of the genus *Lactobacillus*. Helveticin J, produced by *Lactobacillus helveticus* 481 and lacticin B, produced by *Lactobacillus acidophilus* are included in this group.

Class IV bacteriocins consist of complex bacteriocins comprised of protein plus one or more chemical moieties (lipid, carbohydrate) that is required for biological activity.

2.1.3 Leucocin A

Leucocin A is a class IIa bacteriocin that is produced by *Leuconostoc gelidum* UAL187, which was isolated from vacuum-packaged meat (Hastings *et al.* 1991). The production of bacteriocin-like substances by *Leuconostoc* spp. was reported by (Harding and Shaw (1990) and Orberg and Sandine (1984). Furthermore, Harding and Shaw (1990) reported that a bacteriocin that was isolated from *Leuconostoc gelidum* inhibited a broad spectrum of LAB, meat spoilage bacteria and the foodborne pathogen *L. monocytogenes*. It was also reported to inhibit strains of *Enterococcus faecalis* (Felix *et al.* 1994; Hastings *et al.* 1991). Leucocin A is a heat-stable 61 amino acid prepeptide that is encoded on a 7.6 MDa plasmid. After cleavage of the leader peptide the mature bacteriocin consists of 37 amino acids (Hastings *et al.* 1991). The three-dimensional structure of leucocin A was the first to be reported for class II bacteriocins (Fregeau Gallagher *et al.* 1997). In aqueous solution leucocin A is essentially unstructured: however, in a lipophilic environment leucocin A forms an α-helix with amino acids residues 17 to 31. Residues 2 to 16 form a three-stranded antiparallel β-sheet domain, anchored by the disulfide bridge between Cys9 and Cys14 (Fregeau Gallagher *et al.* 1997).

Mesentericin Y105 is a bacteriocin that was isolated from *Leuconostoc mesenteroides* by Hechard *et al.* (1992). It differs from leucocin A by only two amino acid residues: alanine in place of Phe22 and isoleucine in place of Val26. In addition to mesentericin Y105, there are other bacteriocins that strongly resemble leucocin A including leucocin A-TA33a from *L. mesenteroides* TA33a (Papathanasopoulos *et al.* 1997) and leucocin B-TA11a from *L. carnosum* TA11a (Felix *et al.* 1994). These bacteriocins are identical to

leucocin A but they have small differences in their 24 amino acid N-terminal extensions. This raises some interesting questions related to the widespread distribution of identical or similar bacteriocins in nature.

The use of *L. gelidum* as a preservative culture in processed meat is limited because this microorganism is a strong gas producer and it produces dextran (slime) from sucrose that is commonly used in the cure of processed meats (Shaw and Harding 1989); however, leucocin A could be delivered to food systems using the general secretory pathway in a food-grade (genetically modified) organism (Stiles 1993).

2.1.4 Brochocin-C

Brochocin-C is a two peptide, class IIb heat stable bacteriocin produced by *Brochothrix campestris* ATCC 43754, it was originally discovered by Siragusa and Cutter (1993). It was partially purified and characterized by McCormick *et al.* (1998). *B. campestris* was isolated from soil and grass (Talon *et al.* 1988) but because of its close relationship with *Brochothrix thermosphacta*, a spoilage organism of chill-stored vacuum or in modified-atmosphere packaged meat, it can be assumed it may be present in meats (Gao 2001).

The inhibitory spectrum of brochocin-C is comparable to that of nisin (Gao *et al.* 1999) and includes a wide range of Gram-positive bacteria such as the closely related meat spoilage organism *Brochothrix thermosphacta*, the foodborne pathogen *L. monocytogenes* and *Clostridium botulinum* spores (Hurst 1972; McCormick *et al.* 1998). Experiments conducted by Gao *et al.* (1999) showed that brochocin-C is effective against Gram-negative bacteria if the outer membrane is previously disrupted (Gao *et al.* 1999). Brochocin-C consists of two hydrophobic peptides. The mature peptides of Brochocin-C

(brochocin-A and brochocin-B) consist of 59 amino acid residues with a molecular weight of 5,244 Da. and 43 amino acids with a molecular weight of 3,944 Da., respectively. The component peptides A and B have no activity on their own and require the presence of both peptides to be active (McCormick *et al.* 1998).

Table 2 Bacteriocins and their producer strains

Bacteriocin	Classification	Producer strain	Reference for purification
Nisin	Class I (Lantibiotics)	*Lactococcus lactis* ATCC 11454	Purchased from Aplin and Barrett
Leucocin A	Class IIa	*Leuconostoc gelidum* UAL187	Hastings *et al.* 1991
Brochocin-C	Class IIb	*Brochothrix campestis* ATCC 43754	McCormick *et al.* 1998

ATCC - American Type Culture Collection, U.S.A.
UAL - University of Alberta Food Microbiology Lactic Acid Bacteria culture collection

2.2 Potential of LAB bacteriocins in food preservation.

Fermentation of various foods by LAB is one of the oldest forms of biopreservation practiced by mankind. The use of these bacteria and their metabolites is generally accepted by consumers as something "natural" and "health-promoting" (Montville and Winkowski 1997). They play an important role as starter cultures in the manufacture of dairy, meat and vegetable products. Their most important contribution to fermented products besides contributing to the characteristic taste and aroma is food preservation. In most cases the fermentation produces a "new food" with characteristics that are very different to the raw material, for example the transition from milk to cheese. Although

these fermentations occur naturally, it is only in recent years that specific starter cultures have been added to produce the fermented food.

Among the substances produced by LAB that potentially could inhibit growth of other microorganisms are lactic acid and acetic acids, hydrogen peroxide, other compounds such as diacetyl and reuterin, and bacteriocins.

Bacteriocins have been added directly to foods such as processed cheese to prevent spoilage due to gas production of clostridia and to enhance safety by the inhibition of the outgrowth of endospores of *Clostridium botulinum* and the growth of *L. monocytogenes*. Nisin is used mainly to prevent the outgrowth of spores of *C. botulinum*, *Bacillus stearothermophilus* and *Bacillus cereus* in a wide variety of foods, that including canned vegetables, pasteurized egg products and processed cheese (Delves-Broughton 1990). Bacteriocins from LAB have been used to reduce *L. monocytogenes* populations in fermented meats (De Martinis and Franco 1998; Ganzle and Hammes 1996; Schillinger *et al.* 1991; Villani *et al.* 1997). Nisin alone or in combination with small quantities of nitrites has been used to prevent the growth of *C. botulinum* in meats (Rayman *et al.* 1981, 1983) instead of the use of nitrates/nitrites alone. There has been pressure by Government authorities to limit and reduce the amount of nitrate/nitrite added to foods because of the risk that under certain conditions they react with amines from the meat to form nitrosamines that are known carcinogens. However, there are limitations to the use of nisin in raw meat. Rose *et al.* (2003) showed that nisin is inactivated by glutathione that is present in raw meat; nevertheless, nisin can be used in processed (heat-treated) meats under certain conditions (Rose *et al.* 2003). Because of these difficulties in the use

of nisin in raw meat, other LAB bacteriocins have been studied for this purpose. Enterocins, leucocin A, sakacins and carnobactericins A and B prolong the shelf life of fresh meat (Cleveland *et al.* 2001). Pediocin AcH has been used to inhibit foodborne pathogens in ground pork with good initial inhibition but it loses activity over time apparently due to protease degradation (Murray and Richard 1997). It has also been demonstrated that pediocin can be used in combination with other preservatives such as diacetate, lactate and nitrite to inhibit *L. monocytogenes* in turkey slurries (Schlyter *et al.* 1993).

Nisin was also found to be an effective inhibitor of spoilage bacteria in beer and wine fermentations. The broad spectrum class IIa bacteriocin, pediocin AcH, that is produced by *Pediococcus acidilactici* PA-1 has been expressed in *Saccharomyces cerevisiae* for use in wine preservation (Schoeman *et al.* 1999).

Nisin and pediocin have been used in food packaging materials to inhibit *L. monocytogenes*. Teerakarn *et al.* (2000) inoculated some lunch meat with *Listeria* and vacuum packed it inside a soy-based plastic impregnated with nisin showed a drop of 10 percent of the starting *Listeria* count over a period of three weeks. Pediocin powder was used in packaging films during 12 weeks storage at 4°C on meats and poultry and a completely inhibit growth of inoculated *L. monocytogenes* in meats and poultry through 12-week storage at 4°C was found (Ming *et al.* 1997).

Other experiments showed that nisin could be used to inhibit growth of *L. monocytogenes* on poultry skin during refrigerated storage (Mahadeo and Tatini 1994).

Leisner *et al.* (1996) showed that by adding *L. gelidum* UAL187, the leucocin A producer to chill-stored, vacuum-packaged beef inoculated with *Lactobacillus sakei* 1218 (a strain that produces a distinct sulfide odor in meat) spoilage was delayed for up to 8 weeks of refrigerated storage.

2.3 Bacteriocin Resistance

Resistance of microorganisms that are normally sensitive to bacteriocins is a concern for the use of bacteriocins as biopreservatives because reduced sensitivity or resistance to bacteriocins may compromise the antibacterial efficiency of these peptides. Nisin resistance has been reported in *Streptococcus bovis* (Mantovani and Russell 2001), vegetative cells and spores of *C. botulinum* (Mazzotta *et al.* 1997) and *L. monocytogenes* (Gravesen *et al.* 2001, 2002b; Harris *et al* 1989; Mazzotta and Montville 1997; Ming and Daeschel 1993; Murray and Richard 1997). *L. monocytogenes* can develop spontaneous resistance to nisin at a frequency of 10^{-6} to 10^{-7} when nisin is added to the growth medium at high concentration (Davies and Adams 1994). Even higher frequencies of resistance at 10^{-2} to 10^{-5} have been reported at nisin concentrations between 100 and 500 IU/ml (Boutterfroy and Millière 2000). Based on different strains having different sensitivities and resistance frequencies it was concluded that nisin resistance in *L. monocytogenes* may be strain-dependent (Bouttefroy and Millière 2000)

Resistance is reported to be spontaneous or it occurs without prior exposure to bacteriocins (Ennahar *et al.* 2000a; Hurst 1981; Larsen and Nørrung 1993; Rekhif *et al.* 1994). Bacteriocin-sensitive subpopulations include potentially tolerant or resistant cells

with structural modifications or at least with a high predisposition to such modifications that emerge after exposure to bacteriocin. (Noerlis and Ray 1993; Ray 1993).

Resistance to class II bacteriocins has also been studied. In some reports the emergence of resistant *L. monocytogenes* cells in a population occurs at a higher frequency with these bacteriocins than resistance to nisin (Rasch and Knøchel 1998). Rasch and Knøchel (1998) examined 381 strains of *L. monocytogenes* and found twenty strains that were resistant to pediocin PA-1 and 34 strains that they classified as partially resistant because they were only inhibited at the highest concentration of pediocin tested (1,600 AU ml^{-1}). Cross-resistance between different class IIa bacteriocins has also been reported (Dykes and Hastings 1998; Ramnath *et al.* 2000; Rasch and Knøchel 1998), indicating an identical or similar resistance mechanism among bacteriocins.

Mechanisms contributing to resistance appear to be complex and involve various structural and physiological changes in the bacterial cell. Physiological changes in the cytoplasmic membrane of resistant target cells have been investigated (Crandall and Montville 1998; Mazzotta *et al.* 1997; Ming *et al.* 1993). It has been reported that resistant strains of *L. monocytogenes* have a more rigid membrane, usually with a lower C15:C17 fatty acid ratio (Mazzotta and Montville 1997; Ming *et al.* 1993). In addition to changes in the composition of the cytoplasmic membrane, Davies *et al.* (1996) showed that without a cell wall, nisin resistant *L. monocytogenes* mutants lost the resistance. Crandall and Montville (1998) showed that *L. monocytogenes* required divalent cations ($MgSO_4$, $MgCl_2$, $CaCl_2$, $MnSO_4$, or $BaCl_2$) to resist the inhibitory effect of nisin. Vadyvaloo *et al.* (2002) reported an association between increased amounts of

unsaturated and short-acyl-chain phospholipids of phosphatidylglycerol (PGs) in the resistant *L. monocytogenes* strains tested. Alteration in membrane phospholipids toward PGs containing shorter, unsaturated acyl chains suggests that resistant strains have cells with a more fluid membrane (Vadyvaloo *et al.* 2002). Factors under the influence of the σ^{54} factor have been involved in bacteriocin resistance. Robichon *et al.* (1997) studied the mode of action of mesentericin Y105 (a bacteriocin that is closely related to leucocin A) and denoted as a bacteriocin that is active against *L. monocytogenes*. Mutants resistant to mesentericin Y105 resulted from the insertion of a transposon into a the gene *rpoN* that encodes a protein with homology to putative σ^{54} factor (Robichon *et al.* 1997).

Other studies on bacteriocin resistance focused on the influence of σ^{54} factor (Robichon *et al.* 1997) and σ^{54}-dependent genes, specifically the mannose phosphotransferase system (PTS) permease (Dalet *et al.* 2000) and the mannose PTS enzyme IIAB component of *L. monocytogenes* sensitive to leucocin A (Ramnath *et al.* 2000). The up-regulation of a ß-glucoside-specific PTS has been reported in pediocin-resistant *L. monocytogenes* (Gravesen *et al.* 2000). Gravesen *et al.* (2002a) considered that resistance to IIa bacteriocins is acquired through one general mechanism in *L. monocytogenes* and at least some other Gram-positive organisms. This mechanism is characterized by prevention of EII^{Man} synthesis and up-regulation of EII^{Bgl} and the phospho-ß-glucosidase. Up-regulated EII^{Bgl} and phospho-ß-glucosidase expression is not a direct cause of resistance, but it is presumably a regulatory consequence of the lack of *mptACD* expression. Prevention of *mpt* expression directly confers bacteriocin resistance (Gravesen *et al.* 2002a).

Different strategies to reduce the selection of resistant strains have been proposed, including the simultaneous use of more than one bacteriocin from the same or different bacteriocin classes and the use of other physical and/or chemical preservation methods (Hanlin *et al.* 1993; Stevens *et al.* 1992), e. g., by lowering the pH or adding NaCl (Mazzotta *et al.* 1997; Schillinger *et al.* 1998)

2.4 Environmental stress

Food manufacturing, distribution and storage rely on a variety of stresses or "hurdles" that either inhibit or inactivate contaminating microorganisms in the food system. "The stress adaptation phenomenon" is defined as the exposure to a determined stress that increases the resistance to subsequent exposures to the same stress (homologous) or in some instances increases the resistance to other sources of stress (heterologous).

L. monocytogenes is a versatile organism that is capable of growth under many challenging conditions. Examples include refrigeration temperatures, in foods with a high salt content, in acid foods and in the human body where it manages to evade the immune system. Such versatility requires the organism to have the ability to assimilate information about its environment. Lou and Yousef (1997) showed that adaptation of *L monocytogenes* to sublethal doses of ethanol, hydrogen peroxide, salt, acid, heat or nutrient deprivation provided an increase in tolerance to the same lethal factor and cross-protected the organism to different stressors.

The physiological response of bacteria to stress factors is varied and complex. A general strategy used for adaptation to environmental and physical changes involves the reception and transmission of appropriate stimuli by signal transduction (Hoch and Silhavy 1995).

These two-component systems generally consist of a membrane-bound histidine kinase (HK) sensor and a corresponding transcriptional response regulator (RR). When exposed to a specific environmental stimulus, a histidine residue in the HK becomes auto-phosphorylated. Subsequently, the phosphoryl group is transferred to an aspartic acid residue in the RR leading to an alteration in the transcriptional regulation of target genes (Rowan 1999). In *L. monocytogenes* the two-component regulatory system (membrane-bound sensor histidine kinase) is known as *LisR* and *LisK* that monitors a specific environmental parameter and a cytoplasmic response that enables the cell to respond to this environmental parameter (Cotter *et al.* 1999).

Stress proteins that are produced under unfavorable circumstances for the cell are associated with virulence of *L. monocytogenes*, especially during the early stages of intracellular growth (Nair *et al.* 2000; Rouquette *et al.* 1998). Evidence for the role of stress proteins in virulence of *L. monocytogenes* includes the finding that a ClpC ATPase and ClpE that belong to the Hsp-100 family act synergistically in the expression of virulence factors (Rouquette *et al.* 1998; Nair *et al.* 2000). O'Driscoll *et al.* (1996) and Conte *et al.* (2000) also found evidence of increased virulence in mouse model using acid-tolerant mutants of *L. monocytogenes*.

The reaction to stress responses can lead to cross-protection against a range of diverse and apparently unrelated challenges, including resistance to bacteriocins, oxidative stresses and organic acids. Within the food industry, such cross-protective effects may enhance the survival of pathogens and reduce the margin of safety offered by current food processing and preservation techniques (Walsh *et al.* 2001), especially in situations

where the food industry explores levels of lethality that are close to the boundaries (Rowan 1999).

2.5 Sigma factors

Sigma factors are transcriptional factors that regulate the expression of proteins that allow cells to respond to environmental or developmental signals. Sigma factors have been identified in *B. subtilis*, *S. aureus* and *L. monocytogenes* (Becker *et al.* 1998; Wiedmann *et al.* 1998). Because sigma factors function as regulators of stress in Gram-positive bacteria, they have been compared with the better studied σ^S (RpoS) in Gram-negative bacteria, including *Escherichia coli*, *Salmonella*, and *Yersinia* spp.

Sigma^B (σ^B) is a secondary subunit of RNA polymerase that is known to govern a large stress response regulon. The σ^B regulon comprises at least 40 genes related to stress adaptation (Nair *et al.* 2000). Physical signals such as temperature, pH, osmolality and low ATP levels inside the cell also promote σ^B activity (Rowan 1999). The σ^B operon in *L. monocytogenes* contains a number of corresponding regulator-encoding genes such as *rsb U, V, W* and *X* (Becker *et al.* 1998; Wiedmann *et al.* 1998). Sigma^B in *L. monocytogenes* is associated with the ability of this species to use carnitine as an osmoprotectant to enhance survival at low temperatures (Becker *et al.* 1998) and resist acid stress (Ferreira *et al.* 2001; Wiedmann *et al.* 1998) as well as persistence within a host and to host cell infection (Nadon *et al.* 2002). Sue *et al.* (2003) identified and confirmed the σ^B dependence of *L. monocytogenes opuCA*, *lmo1421* and *bsh*. These genes represent general stress-response genes (*opuCA* and *lmo1421* that encode known and putative compatible solute transporter proteins, respectively) and a virulence gene [*bsh*

that encodes a conjugated bile salt hydrolase (BSH)]. They showed that all three genes are expressed under conditions of environmental stress and that expression of *opuCA* and *lmo1421* is induced following exposure to salt stress (Sue *et al.* 2003). The loss of the σ^B function in *L. monocytogenes* σ^B null mutant manifested a 1,000- to 5,000-fold decrease in survival when the mutant was exposed to pH 2.5 (Wiedmann *et al.* 1998).

2.6 Thermotolerance

Heat exposure at lethal levels kills microorganisms by denaturation of enzymes, structural proteins and nucleic acids. Identification of primary sites of heat-induced damage is difficult because of the many changes that heat causes in bacterial cells. Cytoplasmic membrane and structural proteins seem to be major sites of injury of mild (sublethal) heating (Farkas and MohacsiFarkas 1996).

Heat resistance varies widely among different species of bacteria and it is influenced by a variety of factors such as strain variation, previous growth conditions, exposure to heat shock, acid, and other stresses, and composition of the heating menstruum (Doyle *et al.* 2001; Farber and Brown 1990; Fedio and Jackson 1989; Knabel *et al.* 1990; Linton *et al* 1990; Pagan *et al.* 1997). Thermotolerance can increase after exposure to different environmental stresses including heating at sublethal temperatures; viral infections; exposure to chemical compounds such as ethanol, methylating agents, antibiotics (e. g., nalidixic acid), amino acid restrictors, and acid shock (Phan-Thanh *et al.* 2000). Temperatures above the optimum growth temperature are supposed to have a lethal or injurious effect; however, it has been shown that in most microbial species gradual heating or heating for short periods of time at temperatures above the optimum growth

temperature induces higher thermotolerance (Bunning *et al.* 1990, 1992; Pagan *et al.* 1997). It is now believed that these temperatures trigger a physiological response that leads to the synthesis of special proteins known as heat shock proteins (HSP) (Phan-Thanh and Gormon 1997).

Farber and Peterkin (1991) suggested that heat shock response phenomena might explain the discrepancies in thermal inactivation of *L. monocytogenes*. Several workers found that if *L. monocytogenes* is exposed to temperatures of 44 to 48°C, the cells acquire enhanced thermotolerance (Farber and Brown, 1990; Fedio and Jackson, 1989; Knabel *et al.* 1990).

In food products requiring long heating times, such as egg products, or those pasteurized at low temperatures for very long periods of time to retain flavor and texture, such as *sous vide* foods, bacterial pathogens might respond to these mild processing treatments with increased thermotolerance. As a result, there is the risk that some of these microorganisms can survive these thermal processes (Bunning *et al.* 1990; Linton *et al.* 1990).

2.7 Heat Shock Proteins (Hsp)

When a cell is exposed to an environmental stress, it reacts by generating a response that helps it to adapt to the new environmental conditions. In the case of heat stress, the cell produces a group of 12 to 14 different Hsp that are the principal mechanisms to deal with the cellular damage caused by exposure to heat. The principal heat shock proteins in *L. monocytogenes* are chaperones and proteases.

2.7.1.1 Chaperones

Chaperones are proteins that help other proteins fold. They are a major class of the proteins produced after heat stress. The molecular chaperones prevent the aggregation of unfolded proteins inside the cell. Hsp chaperons also recognize and bind to other proteins when the other proteins are in non-native conformations due to protein-denaturing stress or the peptides they comprise have not yet been fully synthesized, folded, assembled, or localized to an appropriate cellular compartment. Binding and/or release of these other proteins is often regulated by association with and/or hydrolysis of nucleotides (Feder and Hofmann 1999). Another function of chaperon proteins is targeting non-native or aggregated proteins for degradation and removal from the cell (Somero 1995). The last two functions are presumed to be the most important for coping with environmental stress. One of the most prominent molecular chaperones is the Hsp70 family known as DnaK. They work together with Hsp40 (DnaJ) and Hsp 60 (GroEL) (Rowan 1999). DnaK and GroEL act as molecular chaperons to protect essential bacterial proteins from heat denaturation. In addition to their role in heat stress, DnaK and GroEL are induced following exposure to other environmental stresses such as low pH, elevated salt concentrations and ethanol indicating a protective role in the general stress response (Gahan *et al.* 2001; Hartke *et al.* 1997; Kilstrup *et al.* 1997)

2.7.1.2 Proteases

Proteases are the second major class of proteins produced following heat stress. When proteins cannot be refolded by chaperones, proteases degrade the damaged proteins and release amino acids for reuse in making new proteins and elimination of proteins that

cannot be repaired (Rowan, 1999). In *Listeria,* the family of Clp proteases plays an important role in heat tolerance. The gene encoding ClpC in *L. monocytogenes* was identified in a transposon mutant that displays sensitivity to low iron conditions (Rouquette *et al.* 1995). Disruption of *clpC* results in reduced thermotolerance and increased sensitivity to high salt and low iron growth conditions. Another member of the family of Clp proteases, ClpP, is required for growth under stress conditions and for virulence of *L. monocytogenes*. Evidence suggests that ClpP may be required for full activity of the essential hemolysin (listeriolysin) virulence factors (Gaillot *et al.* 2000)

2.7.2 Heat shock genes

In Gram-positive bacteria, expression of heat shock genes involves at least three different regulatory mechanisms (Nair *et al.* 2000):

Class I heat shock genes encode heat-specific proteins known as classical chaperones such as GroES, GroEL and DnaK that are controlled by the HrcA repressor. The HrcA repressor exerts its activity through binding to the operator, a very well conserved 9-bp separated by 9-bp spacer inverted repeat called CIRCE (controlling IR of chaperone expression) (Schulz and Schumann 1996; Zuber and Schumann 1994).

The majority of the proteins induced by heat stress belong to the Class II genes, also known as general stress proteins (GSP). These GSPs are also induced by salt stress, exposure to ethanol or starvation for glucose, phosphate and oxygen (Hecker and Völker 1990). These genes constitute a large stress and stationary-phase regulon (Boylan *et al.* 1993; Völker *et al.* 1994) and they are dependent on σ^B factor for transcription.

The Class III gene group includes genes that encode ATP-dependent proteases namely ClpP, ClpC and ClpE. The ClpP operon consists of four genes (*orf1, orf2, orf3* and *clpC*), and its expression is increased at 42°C (Rouquette *et al.* 1996). Nair *et al.* (2000) demonstrated that these genes are negatively controlled by the Class III stress gene repressor (CtsR) homologue of the *B. subtilis* repressor.

Class IV genes include stress response genes that are expressed independent of HrcA, σ^B or CtsR. Their regulatory mechanisms remain to be identified. There is growing evidence to indicate that the ClpC and ClpE Hsp100 ATPases and the recently identified ClpP proteolytic subunit play an important role in stress survival, growth at high temperature and virulence in *L. monocytogenes* (Nair *et al.* 2000; Rouquette *et al.* 1996).

2.8 Salt resistance

The ability of *L. monocytogenes* to tolerate salt (NaCl) is of particular importance, because this pathogen is often exposed to such environments for food preservation. This microorganism can survive relatively high salt concentrations as high as 10% NaCl (McClure *et al.* 1989). This high degree of adaptability is one reason for the difficulty experienced in controlling this pathogen in a number of food products because treatments used in food processing and preservation often utilize stressing agents and other parameters to which *L. monocytogenes* is resistant. Salt is one of the most commonly used agents for food preservation, allowing extended storage time because of the reduced water activity. However, *L. monocytogenes* is frequently isolated from foods containing high quantities of salt, such as smoked salmon (Fonnesbech Vogel *et al.* 2001) and in cheese brining systems (Larson *et al.* 1999).

A better knowledge of the adaptive mechanisms of *L. monocytogenes* to salt stress could lead to better control and prevention of this pathogen in food processing.

When bacterial cells are exposed to high salt concentrations, the cells lose water due to osmosis but solutes inside the cell cannot readily move across the membrane because of this phenomenon, cell shrinkage and in extreme cases plasmolysis of the cell can occur. Microbial cells respond to osmotic stress by changes in lipid and protein components of the cytoplasmic membrane, synthesis of some proteins such as transport proteins and adjustment of cytoplasmic water activity by organic solutes synthesized by the cell or accumulated from the external environment. Some bacteria such as the halophiles can adjust their cytoplasmic water by accumulating KCl to high intracellular concentrations but with the disadvantage of an altered internal environment. As a result, halophiles require high salt concentrations (frequently K+) for most biochemical reactions. This salt-in strategy employed by halophiles limits them to habitats with high salt concentrations because low salt concentration in the surrounding environment would lead to cell disintegration (Britton *et al.* 1998; Dennis and Shimmin 1997).

It has been shown that *L. monocytogenes* responds to elevated osmolarity by a bi-phasic response in which increased levels of K^+ (and its counter-ion glutamate) accumulate as a primary response (Galinski and Trüper 1994), followed by a dramatic increase of compatible solutes (osmolytes) through osmotic activation of their transport from the medium rather than through biosynthesis (Sleator and Hill 2002). In general, compatible solutes are small, highly soluble molecules that do not carry net charge at physiological pH (Galinski and Trüper 1994). They act in the cytosol by counterbalancing the external

osmolarity and they do not interact with proteins, which facilitates their accumulation to high intracellular concentrations without adversely affecting vital cellular processes such as DNA repair, DNA–protein interactions and the cellular metabolic machinery (Strom and Kaasen 1993). Among the compatible solutes, the amino acid proline, the trimethyl ammonium compound glycine betaine (trimethylglycine), and the structurally related trimethyl amino acid γ-*N*-trimethyl aminobutyrate (L-carnitine) are the major osmolytes found in *L. monocytogenes* (Beumer *et al.* 1994; Ko *et al.* 1994; Smith 1996). In addition to their role as osmotic protectors, compatible solutes protect enzyme function, provide protection against salinity, high temperature, freeze-thaw treatment and even drying (Lippert and Galinski 1992; Welsh 2000).

Sleator *et al.* (1999) presented molecular evidence for osmolyte uptake in *L. monocytogenes*. They found that betaine uptake is regulated by BetL, an integral membrane protein that functions as a porter to allow the passage of betaine through the cytoplasmic membrane. The *betL* gene possesses a consensus σ^B-dependent promotor binding site suggesting that at that at least a component of betaine uptake by BetL is regulated at the transcription level (Sleator *et al.* 1999, 2003). Additionally, the four genes encoding OpuC, the principal carnitine uptake system in *Listeria*, were independently identified (Fraser *et al.* 2000; Sleator *et al.* 2001). Mutating *opuC* results in a significant reduction in both betaine and carnitine uptake, particularly at elevated osmolarity (Sleator *et al.* 2001).

2.9 Acid Resistance

The use of organic acids in food preservation, either by fermentation or by intentional addition is an important and widespread mechanism for controlling foodborne pathogens. *L. monocytogenes* is more acid tolerant than most foodborne pathogens (Barker and Park 2001), although the sensitivity of the organism to organic acids varies with the nature of the acidulant used (Sorrells *et al.* 1989).

Strains of *L. monocytogenes* differ in their basal levels of acid resistance and their physiological state is also a determinant of the acid response (Dykes and Moorhead 2000; Phan-Thanh *et al.* 2000).

Direct action of protons on microbial cells involves the cell wall, the outer structure of cytoplasmic membrane and membrane-bound proteins. The cytoplasmic membrane works as a barrier to protons because with their charge they penetrate the membrane poorly but at extremely low external pH (pH_o), protons leak across the membrane and acidify the internal pH (pH_i). Proteins are the most likely component of the cell to be affected by low pH, altering the secondary and tertiary structure and as a consequence loose activity and function. In contrast, uncharged acid molecules diffuse rapidly though the cytoplasmic membrane if they are lipid-soluble. Once the uncharged molecules enter the cytoplasm, the neutral pH inside the cell induces the dissociation of the acids into charged anions and the protons that concentrate in the cytoplasm lower the internal pH, causing denaturation of enzymes and nucleic acids (Booth *et al* 2002).

Lipid solubility is important to consider in choosing an organic acid as a food preservative, because some organic acids such citric, malic, fumaric and tartaric acids are

not lipophilic thus these acids do not diffuse through lipid membranes; whereas, acetic acid is lipid-soluble and diffuses rapidly through the membrane (Doyle 1999).

Survival of microorganisms in extreme acid environments can be due to adaptation to sublethal levels of acidity. This phenomenon is known as an acid-tolerance response (ATR) and it has been well-characterized in *E. coli* (Heyde and Portalier 1990) and *Salmonella enterica* serovar Typhimurium (Foster and Hall 1991), in which exposure to sublethal pH induces the expression of numerous acid-shock proteins (ASPs) that promote bacterial survival in extreme acid environments (Herbert and Foster 2001).

L. monocytogenes responds to acid stress by two principal mechanisms: (i) the Glutamate Decarboxylase acid resistance system (GAD) and (ii) the F_0F_1-ATPase resistance mechanism. When the cell is exposed to low pH, glutamate is taken up by a specific transporter and cytoplasmic decarboxylation results in the production of γ-aminobutyrate (GABA). This is exported from the cell via an antiporter with the consumption of a proton. The net effect of this reaction is to increase the alkalinity of the cytoplasmic compartment along with a slight increase in the extracellular pH (Cotter *et al.* 2001; Small and Waterman 1998). At least two proteins are involved in the GAD system: a glutamate/GABA antiporter in the cell membrane; and a cytoplasmic glutamate decarboxylase to convert glutamate plus a proton to γ-aminobutyrate and CO_2 (Cotter *et al.* 2001).

The F_0F_1-ATPase resistance mechanism increases the intracellular pH under acidic conditions. This is a multisubunit enzyme consisting of a catalytic portion (F_1), which may synthesize or hydrolyze ATP and an integral membrane portion (F_0), which

functions as a membranous channel for proton translocation (Hill *et al.* 2002). The role of the F_0F_1-ATPase in *Listeria* is to synthesize ATP aerobically, as a result of protons passing into the cell, and to generate a proton motive force (PMF) anaerobically, in association with the expulsion of protons.

3 Material and Methods

3.1 Media and reagents

Bacterial strains used in this study are listed in Table 3. Strains of *Listeria monocytogenes* were grown in Tryptic Soy Broth (Difco Laboratories Inc., Becton Dickinson Microbiology Systems, Sparks, Maryland) supplemented with 0.6% Yeast Extract (TSBYE; Difco) at 35°C without agitation. *Brochothrix campestris* ATCC 43754, *Carnobacterium divergens* UAL9, *Lactococcus lactis* ATCC 11454, *Leuconostoc gelidum* UAL187, *Pediococcus acidilactici* PAC 1.0 were grown in APT (All Purpose Tween, Difco) broth at 25°C without agitation. Stock cultures were stored at -70°C in the appropriate media containing 20% glycerol. Cultures were subcultured twice (1% inoculum) and no more than five times in the corresponding broth media before use in experiments.

3.2 Concentration of leucocin A

An inoculum of 10 ml of an overnight culture of *Leuconostoc gelidum* UAL187 was added to 500 ml of APT broth. After incubation at 25°C for 24 h, cells were harvested by centrifugation at 6,000 x *g* for 30 min at 4°C. The supernatant was loaded onto a 6 x 32 cm Amberlite XAD-8 column (BDH, Darmstadt, Germany). The column was washed with 1 liter of 0.1% trifluoroacetic acid (TFA) and successively with 1 liter solutions of 20, 40 , 60 and 80% ethanol in 0.1% TFA. The fractions with inhibitory activity were pooled and concentrated with a rotary evaporator (≤ 30°C) and freeze dried for 48 h. The fractions were resuspended in 0.1% TFA, and filter-sterilized through a low protein

binding filter (Millex GV 0.22 µm, Millipore SA. Molsheim, France). The level of the leucocin A in solution was determined by spot-on-lawn assay (Ahn and Stiles 1990) using *Carnobacterium piscicola* UAL9 as the indicator strain.

Table 3. Bacterial strains used in this study.

Organism	Source/Reference
Brochothrix campestris 43754	ATCC
Carnobacterium piscicola UAL9	UAL
Lactococcus lactis 11454	ATCC
Leuconostoc gelidum UAL187	Hastings *et al.* 1991
Listeria monocytogenes 15313	ATCC
Listeria monocytogenes Scott A	ATCC
Pediococcus acidilactici PAC 1.0	Vandenbergh [a]

a. P. A. Vandenbergh (Quest International, Sarasota, U.S.A.)

3.3 Bacteriocin resuspension

A stock solution of purified nisin (1 mg/ml, equivalent to 50 IU/ml) purchased from Aplin and Barrett Ltd. (Dorset, United Kingdom) was prepared in 0.02 N HCl and filter-sterilized with a Millex GV 0.22 µm (Millipore) filter. Concentrated and lyophilized leucocin A was resuspended in 10 ml of 0.1% TFA, for use as a stock solution. Stock solutions were stored at -70°C. Brochocin-C was concentrated on an Amberlite XAD-8 column (BDH, Darmstadt, Germany). Active fractions were pooled and concentrated in a rotary evaporator at 55°C and freeze-dried. The active fractions were resuspended in 0.1% TFA, and filter sterilized. Spot-on-lawn assay was performed to determine the

relative brochocin-C concentration against *L. monocytogenes* Scott A and *C. divergens* UAL9.

3.3.1 Assay for bacteriocin activity

Inhibitory activity of brochocin-C, leucocin A and nisin were determined using the spot-on-lawn and deferred inhibition assays described by Ahn and Stiles (1990) and Barefoot and Klaenhammer (1983). Serial, two-fold dilutions (1:1) of cell supernatant (heat treated at 60°C for 30 min) were prepared in sterile Milli-Q water and 10 µl of each dilution was spotted onto an APT agar plate, allowed to dry and overlayered with 6 ml of soft APT agar (0.75% agar) at 45°C inoculated with 1% of the indicator strain. Activity was calculated as the reciprocal of the highest dilution that causes a clear inhibition of the indicator organisms *L. monocytogenes* Scott A and *C. divergens* UAL9 and was expressed as arbitrary units (AU) against each indicator organism. Confirmation of the proteinaceous nature of the antimicrobial compound in the zones of inhibition was done by spotting 5 µl of pronase E from *Streptomyces griseus*, type XIV (1 mg/ml, Sigma P-6911) adjacent to one of the previously dried spots of the bacteriocin solution prior to incubation.

In the deferred inhibition assay (Barefoot and Klaenhammer 1983), the bacteriocin producer organisms were inoculated onto APT agar using a Cathra replicating inoculator (KVL Laboratories, Cambridge, Ontario, Canada) and incubated at 25°C for 24 h. After incubation, the plates were overlayered with a 1% inoculum of the *L. monocytogenes* strain in 6 ml of soft APT agar (0.75% agar). After 24 h of incubation at 35°C, zones of inhibition surrounding the producer strain were recorded as a positive for production of

inhibitory substance and the indicator strains used in the bacterial lawn were recorded as sensitive to the inhibitory substance.

3.4 Isolation and frequency of bacteriocin-resistant strains of *L. monocytogenes*

For the isolation of brochocin-C resistant strains, bacteriocin was added to TSBYE agar at 50°C to give final concentrations of brochocin-C of 100, 200, 400, 800 and 1,600 AU/ml and inoculated with 10^8 to 10^9 cells of *L. monocytogenes* Scott A and ATCC 15313. The mixture was poured onto sterile Petri dishes and incubated at 30°C for 48 h. The frequency of spontaneous resistance was determined by comparing the number of colonies at each brochocin-C concentration with the number of colonies in the original inoculum (Gao 2001). Nisin-resistant cells were obtained following the same procedure. Individual colonies obtained from exposure to bacteriocins were picked and subcultured in fresh TSBYE broth with the corresponding bacteriocin (500 AU/ml) to promote and maintain the resistance character.

An inoculum of 10^3 to 10^4 cells of *L. monocytogenes* ATCC 15313 or Scott A was added per ml to a tube of TSBYE containing 500 AU of leucocin A per ml. After 18 h of incubation at 30°C, 1 ml of the culture was centrifuged at 16,000 x *g* for 5 min and washed twice with sterile, (0.1%) peptone water; then used as indicator lawns in spot-on-lawn assays.

3.5 Stability of the resistance character and cross-resistance

Resistant strains of *L. monocytogenes* were subcultured ten times in TSBYE without bacteriocin at 30°C. After each subculture the cells were harvested and washed twice

with phosphate buffered saline (PBS; 0.02 M sodium phosphate buffer with 0.15 M sodium chloride, adjusted to pH 7.4,) and used as an indicator lawn in the deferred inhibition assay (Rekhif *et al.* 1994). Resistance was confirmed by picking and streaking single colonies onto TSBYE agar containing one of the test bacteriocins. Deferred inhibition assays were used to determine cross-resistance. Each bacteriocin-resistant and wild-type strain was tested for resistance to each of the three bacteriocins.

3.6 Random amplification of polymorphic DNA (RAPD) procedure

DNA for RAPD was extracted from *L. monocytogenes* cultures by bead beating, as described by Walter *et al.* (2000). Plates of TSBYE agar were used to prepare a suspension of cells in 1 ml of Milli-Q water. The suspensions were centrifuged at 12,000 x *g* for 3 min. at 5°C and washed with 1 ml of TN150 buffer (10 mM TRIS-HCl, pH 8, 150 mM NaCl). The cells were transferred to sterile tubes containing 0.3 g of zirconium beads (0.1 mm diameter). Samples were shaken in a Mini Bead Beater (BioSpec Products, Inc., Bartlesville, OK) at 5,000 rpm for 3 min. and placed on ice to cool and to allow the zirconium beads to settle to the bottom of the tube. A volume of 500 µl of the DNA suspension was extracted sequentially with 500 µl of saturated phenol and with chloroform-isoamyl alcohol (24:1) mixture. The extracted DNA was centrifuged at 12,000 x *g* for 3 min. at 5°C and mixed with 2 vol. of ethanol (95%) at -20°C and 50 µl of sodium acetate 3 M at -20°C and held overnight at -20°C. The preparations were centrifuged at 12,000 x *g* for 20 min. at 5°C and the pellets were dried by placing the tubes upside down. The dried pellets were dissolved in TE buffer (Tris 10 mM, EDTA 1 mM), 25 µl of RNAse (ribonuclease A) 2 mg/ml (Sigma Chemical, St. Louis, MO

U.S.A.) was added and samples were incubated at 37°C for 1 h. The phenol-chloroform extraction and drying steps were repeated, the pellet was dissolved in 20 µl of TE buffer and 1 µl of the solution was loaded onto an agarose gel (0.7%) to calculate the DNA concentration of each sample.

RAPD analysis was performed as described by Lawrence *et al.* (1993) with some modifications. The previously extracted DNA was used in the amplification mixture. A nucleotide primer M13 (5'-GAGGGTGGCGGTTCT-3') was used in the PCR amplification mixture and consisted of the 4 deoxynucleotide triphosphates (dNTPs) 100 mM, $MgCl_2$ 4 mM, 1.25 U of DNA Taq polymerase (0.1 µl) (Invitrogen Life Technologies, Carlsbad, CA), 10x PCR buffer (100 mM Tris-HCl at pH 8.3 and 500 mM KCl, 9 mM $MgCl_2$, 0.1% gelatin) and sterile Milli-Q water was added to the reaction mixture to bring the final volume to 99.5 µl. PCR was performed with a thermocycler (GeneAmp PCR System 2400; Perkin-Elmer, Norwalk, CT) using 40 cycles of 1 min. at 94°C, 2 min. at 45°C, and 2 min. at 72°C, followed by 5 min. at 72°C. The Taq DNA polymerase (0.5 µl) was added after 2 min. at 94°C in the first cycle. After completion of PCR amplification the samples were held at -20°C for gel electrophoresis. Gel electrophoresis was run by applying 20 µl of amplified DNA and 2 µl of loading dye to a submerged horizontal agarose gel (1.8% w/v; Life Technologies, Inc., Gaithersburg, MD) and 5 µl of ethidium bromide (10 mg/ml) in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). A 100-kb DNA ladder (GibcoBRL®) was included twice in each gel as a molecular weight marker. The gel was loaded into an electrophoresis unit (Bio-Rad) and submerged in TAE buffer. Gels were run at 100 V for 2.5 h by an electrophoresis power

supply (Bio-Rad Powerpac 300). Bands were visualized with UV light with a Gel Doc 1000 and analyzed by Molecular Analyst software (Bio-Rad).

3.7 Resistance to lethal preservation factors

3.7.1 Measurement of acid tolerance

The resistance to low pH was examined with a modification of the protocol described by Lou and Yousef (1997). A 100 µl aliquot of a full growth culture of bacteriocin-resistant and wild-type *L. monocytogenes* culture (*ca.* 10^9 CFU/ml) ATCC 15313 and Scott A was separately mixed with 10 ml of acetic acid (0.1 M, pH 3.6) in test tubes (18 x 150 mm.) During inactivation, samples were taken every 5 h for 25 h at 25°C and a portion (0.1 ml) of the sample, or dilution thereof, was plated onto TSBYE agar. The plates were incubated at 30°C for 48 h and colonies were counted. Experiments were done in triplicate.

3.7.2 Measurement of thermal tolerance

The heat resistance of bacteriocin-resistant *L. monocytogenes* was compared with the wild-type cells at 60°C. A 100 ml volume of fresh TSBYE broth in a 250-ml flask was separately inoculated with 1 ml of fully-grown culture of wild-type and bacteriocin-resistant *L. monocytogenes*. Samples were heated at 60°C in a thermostatically controlled water bath (HAAKE E52, HAAKE Instruments Inc., Saddle Brook, NJ) and stirred with a magnetic stirrer (Bellco Glass Inc., Vineland, NJ). Samples of 0.1 ml were removed at predetermined time intervals and immediately cooled in an ice-water bath. Heat-treated samples were diluted in sterile, 0.1% peptone water for enumeration of appropriate

dilutions and plated on TSBYE agar, and incubated at 30°C for 48 to 72 h prior to enumeration. The $\log_{10}$ number of survivor cells was plotted against time. Each experiment was replicated five times.

3.7.3 Measurement of salt tolerance

Resistant and wild-type *L. monocytogenes* cultures (ca. 10^9 CFU/ml) were added to 10 ml of 20% NaCl in 0.1 M phosphate buffer (PB) at pH 7 and incubated at 25°C. Samples were taken every 50 h for 300 h and 0.1 ml of the sample or dilution thereof was plated on TSBYE agar. Plates were incubated at 30°C for 48 h and colonies were counted. Each experiment was replicated three times.

3.8 Statistical analysis

All microbiological data was converted to log CFU/ml prior to analysis and means were calculated across replicates of the experiments. Differences between treatments were determined using analysis of variance (ANOVA; StatSoft, Inc. 2000 Tulsa, OK). The comparison of the means was done by Tukey's multiple range test (StatSoft, Inc.). Differences were considered significant if $p < 0.05$. Data are means of three replicates except for thermotolerance experiment with five replicates. Error bars represent standard errors of the means.

4 Results

4.1 Bacteriocin activity

Leucocin A produced by *L. gelidum* UAL187 in APT broth was concentrated by XAD-8 hydrophobic interaction chromatography to 6,400 AU/ml and 3,200 AU/ml tested by spot on lawn assay against *L. monocytogenes* Scott A and *C. piscicola* UAL 9, respectively. The relative activity of the nisin and brochocin-C against the same indicators is also shown in Table 4.

Table 4. Bacteriocin concentration determined by spot-on-lawn assay.

Bacteriocin	Indicator strain *L. monocytogenes* Scott A AU/ml	Indicator strain *C. divergens* UAL 9 AU/ml
Leucocin A	6,400	3,200
Brochocin-C[1]	12,800	6,400
Nisin (1 mg ml^{-1})	3,200	1,600

[1] Brochocin-C purified by XAD-8 column by Gao (2001)

4.2 Isolation and frequency of occurrence of bacteriocin-resistant strains of *L. monocytogenes*

4.2.1 Isolation of brochocin-C resistant strains

Frequency of spontaneous resistance of *L. monocytogenes* Scott A and ATCC 15313 to brochocin-C used at different concentrations is shown in Table 3. Both strains had similar frequencies of resistance when they were grown in the presence of 100 AU of brochocin-C but at 200 and 400 AU slight differences were observed in which *L. monocytogenes*

ATCC 15313 had higher frequencies of bacteriocin-resistance. The wild-type *L. monocytogenes* was used as a control and was plated under the same conditions in the absence of brochocin-C produced a confluent lawn of cells.

Table 5. Frequency of resistance of *L. monocytogenes* Scott A and ATCC 15313 to brochocin-C.

Brochocin-C concentration AU/ml	Frequency of resistance Strain Scott A	Strain ATCC 15313
100	2.3×10^{-3}	4.9×10^{-3}
200	1.3×10^{-6}	8.1×10^{-5}
400	6.7×10^{-7}	6.0×10^{-6}

4.2.2 Isolation leucocin A resistant strains

Strains that were resistant to leucocin A were not isolated when the *Listeria* strains were grown in the presence of different concentrations of leucocin A. In three replications of the experiment, there was no growth of the *L. monocytogenes* strains when leucocin A was added at final concentrations of 100, 200, 400, 800, 1,600 AU/ml. As a result, an alternative procedure to isolate leucocin-resistant strains was attempted. *L. monocytogenes* ATCC 15313 and Scott A were inoculated at 10^3 to 10^4 cells per ml into tubes of TSBYE containing 500 AU of leucocin A per ml. After 18 h of incubation at 30°C, 1 ml of the culture was centrifuged at 6,000 x *g* for 5 min. and washed twice with sterile, 0.1% peptone water and used as an indicator lawn in spot-on-lawn assays. Cells resistant to leucocin A 500 AU ml^{-1} were obtained from within the zones of inhibition

using this approach; however, frequencies of resistance to leucocin A could not be determined because of the limitations of the method.

4.2.3 Isolation of nisin resistant strains

Frequency of spontaneous resistance of *L. monocytogenes* Scott A and ATCC 15313 to nisin are shown in Table 6. Both strains showed similar resistance frequencies at the different nisin concentrations. Controls without nisin produced a confluent lawn of cells.

Table 6. Frequency of resistance of *L. monocytogenes* Scott A and ATCC 15313 to nisin.

Nisin concentration AU/ml	Frequency of resistance Strain Scott A	Strain ATCC 15313
100	7.43×10^{-4}	5.45×10^{-4}
200	2.11×10^{-5}	1.01×10^{-5}
400	4.75×10^{-6}	1.31×10^{-5}

4.3 Cross-resistance

The sensitivity of the bacteriocin-resistant and wild-type strains to other bacteriocins is reported in Table 7. Strains resistant to brochocin-C (bro^r) and leucocin A (leu^r were not inhibited by *B. campestris* ATCC 43754 (the brochocin-C producer) and *L. gelidum* UAL187 (leucocin A producer), respectively. Furthermore, they were also resistant to the other class II bacteriocin tested (data not shown). All of the strains tested were sensitive to nisin produced by *L. lactis* ATCC 11454 except for one of the bro^r strains that also

developed resistance to nisin. *L. monocytogenes* Scott A and ATCC 15313 wild-type strains were included as sensitive controls.

Table 7. Cross-resistance of *L. monocytogenes* wild-type, brochocin-resistant and leucocin-resistant strains to bacteriocin-producing LAB by deferred inhibition assay.

Strains of *L. monocytogenes* (Indicator strains)	Bacteriocin producers		
	L. lactis ATCC 11454 (nisin)	*B. campestris* ATCC 43754 (brochocin-C)	*L. gelidum* UAL187 (leucocin A)
15313 wild-type	++	++	++
15313 bro^r	++	--	--
15313 leu^r	++	--	--
Scott A wild-type	++	++	++
Scott A bro^r 1	++	--	--
Scott A bro^r 2	--	--	--
Scott A leu^r	++	--	--

Diameter of the zone of inhibition appearing in the indicator overlay.
++ 10 to 14 mm
+ 5 to 9 mm
-- Less than 5 mm
bro^r *L. monocytogenes* strains resistant to brochocin-C
leu^r *L. monocytogenes* strains resistant to leucocin A

4.4 Stability of the resistance phenotype

The stability of the acquired resistance phenotype was studied by subculturing the resistant strains of *L. monocytogenes* for at least 100 generations of exponential growth in TSBYE broth without added bacteriocin. After subculture for 2, 5 and 10 days, the bacteriocin-resistance phenotype was tested with resistant strains used as indicator lawns in spot-on-lawn assay against the respective bacteriocin. The corresponding wild-type strains were included as sensitive controls.

The stability of brochocin-C resistance in two strains of *L. monocytogenes* is shown in Table 6. Resistant strains derived from single and sequential exposure were stable for the first day of the experiment; however, by day 5, *L. monocytogenes* ATCC 15313 with a single exposure to brochocin-C showed sensitivity and by day 10 both strains with a single exposure to brochocin-C reverted to bacteriocin sensitive. Strains exposed sequentially to brochocin-C in five consecutive subcultures remained resistant to 12,800 AU of brochocin-C per ml for 100 generations.

Table 8. Stability of brochocin-C resistance in strains of *L. monocytogenes* after a single or multiple exposure to brochocin-C determined by spot-on-lawn assay.

Strains of *L. monocytogenes*	Sensitivity to Brochocin-C (AU/ml) Day 1	Day 5	Day 10
15313 bro$^{r\,1}$	----	6,400 (10 mm)	3,200 (10 mm)
Scott A bro$^{r\,1}$	----	----	12,800 (10 mm)
15313 bro$^{r\,2}$	----	----	----
Scott A bro$^{r\,2}$	----	----	----

[1] Resistant strain with single exposure to brochocin-C

[2] Resistant strain with multiple exposures to brochocin-C

---- No zone of inhibition

A second experiment was done to confirm the stability of bro^r by picking and streaking single colonies onto TSBYE agar containing 5,000 AU of brochocin-C, (see Table 7). Isolates exposed once to brochocin-C gradually reverted to being sensitive to brochocin-C; whereas, isolates with sequential exposures retained their resistant character at 100% over 10 days of successive incubation without brochocin-C in the growth medium. Wild-type strains were used as sensitive controls.

Table 9. Stability of brochocin-C resistant phenotype in strains of *L. monocytogenes* ATCC 15313 and Scott A obtained after single and multiple exposures to brochocin-C

Strains of *L. monocytogenes*	% of Brochocin-C resistance		
	Day 1	Day 5	Day 10
15313 wild-type	0	0	0
15313 bro^{r1}	100	64	28
15313 bro^{r2}	100	100	100
Scott A wild-type	0	0	0
Scott A bro^{r1}	100	72	32
Scott A bro^{r2}	100	100	100

[1]Resistance acquired with a single exposure to brochocin-C

[2]Resistance acquired with multiple exposures to brochocin-C

Each data point is the mean of three experiments.

Based on the data shown in Tables 6 and 7 the strains with stable resistance to brochocin-C were selected for use in the lethal preservation experiments.

Stability of leucocin A resistance was determined in the same way by spot-on-lawn assay using leucocin A-resistant strains of *L. monocytogenes* as the indicator lawn (see

Table 10). Zones of inhibition were interpreted as a loss of leucocin A resistance. Strains of *L. monocytogenes* that were isolated after a single exposure to the leucocin A, reverted to being sensitive to leucocin A, whereas strains exposed to leucocin A in 5 successive subcultures retained the resistance character. Similar effect was observed when individual colonies were streaked on TSBYE agar supplemented with leucocin A at 5,000 AU/ml (Table 11). Wild-type strains were used as sensitive controls.

Table 10. Stability of leucocin A resistant strains of *L. monocytogenes* ATCC 15313 and Scott A determined by spot-on-lawn assay

Strains of *L. monocytogenes*	Sensitivity to Leucocin A (AU/ml) Day 1	Day 5	Day 10
15313 leu^{r} [1]	----	1,600 [3]	800
15313 leu^{r} [2]	----	----	----
Scott A leu^{r} [1]	----	800 [3]	400
Scott A leu^{r} [2]	----	----	----

[1] Resistant strain from single exposure to leucocin A

[2] Resistant strains from multiple exposures to leucocin A

[3] diffuse growth inside the zone of inhibition

---- No zone of inhibition

Table 11. Stability of leucocin A resistant strains of *L. monocytogenes* determined by exposure of single colonies on TSBYE agar plates containing 5,000 AU of leucocin A per ml after growth for 100 generations in the absence of leucocin A

Strains of *L. monocytogenes*	% of Leucocin A resistance		
	Day 1	Day 5	Day 10
15313 wild-type	0	0	0
15313 leu^{r} [1]	100	100	0
15313 leu^{r} [2]	100	100	96
Scott A wild-type	0	0	0
Scott A leu^{r} [1]	100	100	0
Scott A leu^{r} [2]	100	100	92

[1] Resistant strain from single exposure to brochocin-C

[2] Resistance strains from multiple exposures to brochocin-C

The stability of nisin resistance was also determined by spot-on-lawn assay (Table 12). In contrast, the nisin resistance characteristic was considered to be transient because nisin resistant strains lost the resistance character after two subcultures in the absence of nisin. Same result was found in each of five attempts to generate strains with stable resistant phenotype.

Table 12. Stability of nisin resistant strains of *L. monocytogenes* determined by spot-on-lawn assay

Strains of *L. monocytogenes*	Sensitivity to nisin (AU/ml) Day 1	Day 2	Day 3	Day 10
15313 nisin^{r} [1]	----	400	100	100
Scott A nisin^{r} [1]	----	400	200	100
15313 bro^{r} [2]	----	----	----	----

[1] Resistant strain from single exposure to nisin

[2] brochocin-C-resistant strain with nisin resistance

---- No zone of inhibition (not resistant)

4.5 Genetic validation of strain identity

Random amplification of polymorphic DNA technique was used to confirm the identity of bacteriocin-resistant strains compared with their parent strains. Strains with the same RAPD profiles were considered to be the same bacterial strain.

As shown in Figure 1, the strains tested in lanes 2, 3 and 5 had similar RAPD profile as that of the wild-type strain in lane 1. Strain in lane 4 had a different profile than the wild-type and was discarded. Only the strains with the confirmed RAPD profiles were used in subsequent studies. RAPD tests were also done on strains of *L. monocytogenes* Scott A resistant to bacteriocins and wild-type. The data shown in Figure 2 indicate that the RAPD profiles for the strains tested in lanes 3, 4, 5, 6, and 7 were similar to the profile for the parent strain of Scott A shown in lane 2. Therefore they could all be considered to be resistant derivatives of Scott A strain with different sensitivity to bacteriocins.

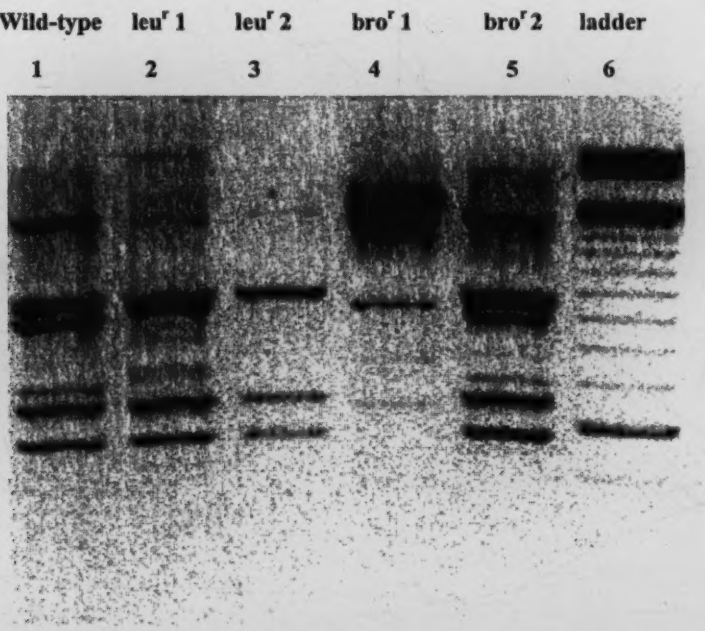

Figure 1. RAPD profiles of *L. monocytogenes* 15313 obtained with primer M13 after RAPD analysis in a gel containing 1.8% agarose. Lane 1 is the wild-type strain, lanes 2 and 3 are leu^r strains, lanes 4 and 5 are bro^r strains and lane 6 is a marker lane with a 100-bp DNA ladder.

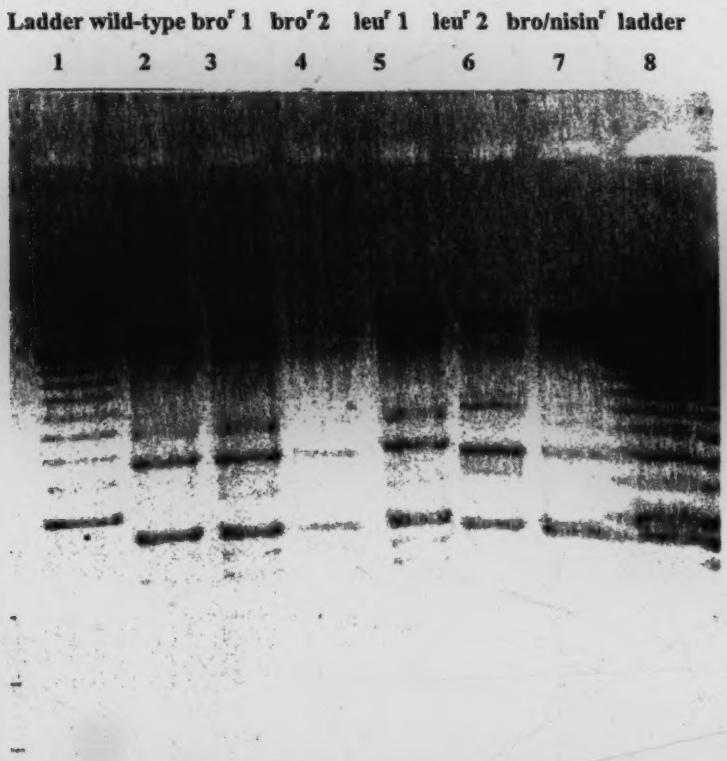

Figure 2. RAPD profile of *L. monocytogenes* Scott A obtained with primer M13 after RAPD analysis in a gel containing 1.8% agarose. Lanes 1 and 8 are marker lanes with 100-bp DNA ladders, lane 2 is the wild-type strain, lanes 3 and 4 are bro^r strains, lanes 5 and 6 are leu^r strains; lane 7 is a bro^r and nisin resistant strain.

4.6 Heat-tolerance

Heat-tolerance was studied in bacteriocin-resistant and wild-type strains in TSBYE. A 1% inoculum of each fully-grown culture of *L. monocytogenes* was heated at 60°C and samples were collected at previously determined time intervals. Data obtained in 5 repetitions of the heat-tolerance experiment were subjected to ANOVA analysis using Statistical software (StatSoft, Inc. 2000 Tulsa, OK, USA).

4.6.1 *L. monocytogenes* ATCC 15313

Data for the heat-tolerance of *L. monocytogenes* ATCC 15313 are shown in Figure 3. A significant difference ($p<0.05$) in heat-tolerance between the wild-type strain and the leucocin A resistant was found. The leu^r strain of *L. monocytogenes* 15313 ATCC 15313 was more thermotolerant than the wild-type and the bro^r strains.

4.6.2 *L. monocytogenes* Scott A

Thermotolerance data for *L. monocytogenes* Scott A are shown in Figure 4. No significant differences ($p>0.05$) were detected in heat resistance between the wild-type and the bacteriocin-resistant strains.

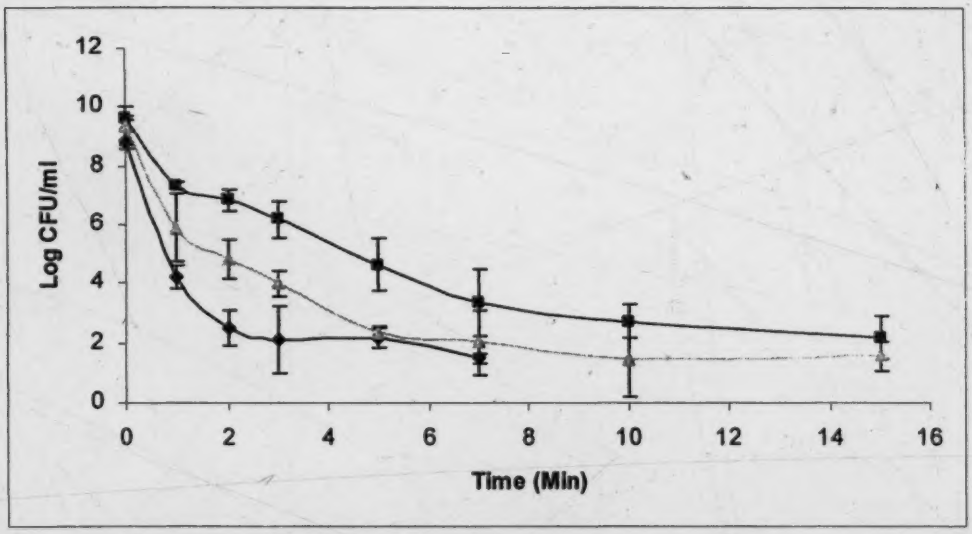

Figure 3. Heat-tolerance of *L. monocytogenes* 15313 wild-type (♦), 15313 Leu^r (■) and 15313 bro^r (▲) strains at 60°C. Data represents the mean of five replications.

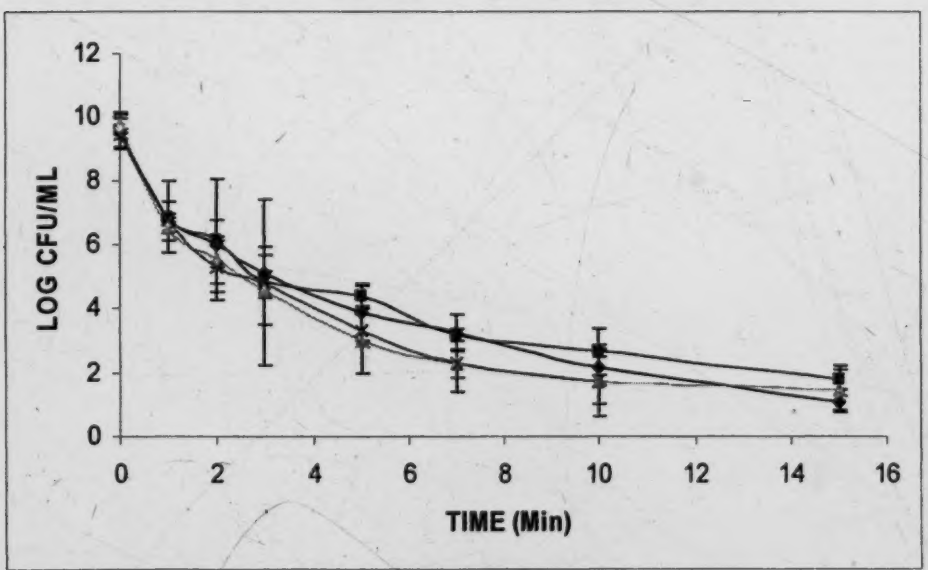

Figure 4. Heat-tolerance of *L. monocytogenes* Scott A wild-type (♦), Leu^r (■), bro^r (▲) and bro-nis^r (×) resistant strains at 60°C. Data represents the mean of five replications

4.7 NaCl-Tolerance

The sensitivity of *L. monocytogenes* strains to salt were studied in TSBYE supplemented with 20% NaCl. Samples were taken at predetermined time intervals and plated on TSBYE agar to determine the bacterial count.

4.7.1 *L. monocytogenes* ATCC 15313

ANOVA showed that the different strains of *L. monocytogenes* ATCC 15313 had different salt-tolerance characteristics. The data analyzed by Tukey's test revealed that 15313 leu^r was more salt-tolerant than the wild-type and 15313 bro^r. No significant difference ($p>0.05$) was found in the salt tolerance of wild-type and 15313 bro^r strains (Figure 5).

4.7.2 *L. monocytogenes* Scott A

L. monocytogenes Scott A strains showed no significant difference in salt-tolerance between wild-type and bacteriocin-resistant strains; however, means analyzed by Tukey's test shown significant difference ($p<0.05$) among leu^r and bro^r strains. Leu^r strains were more NaCl-tolerant than bro^r strains (Figure 6).

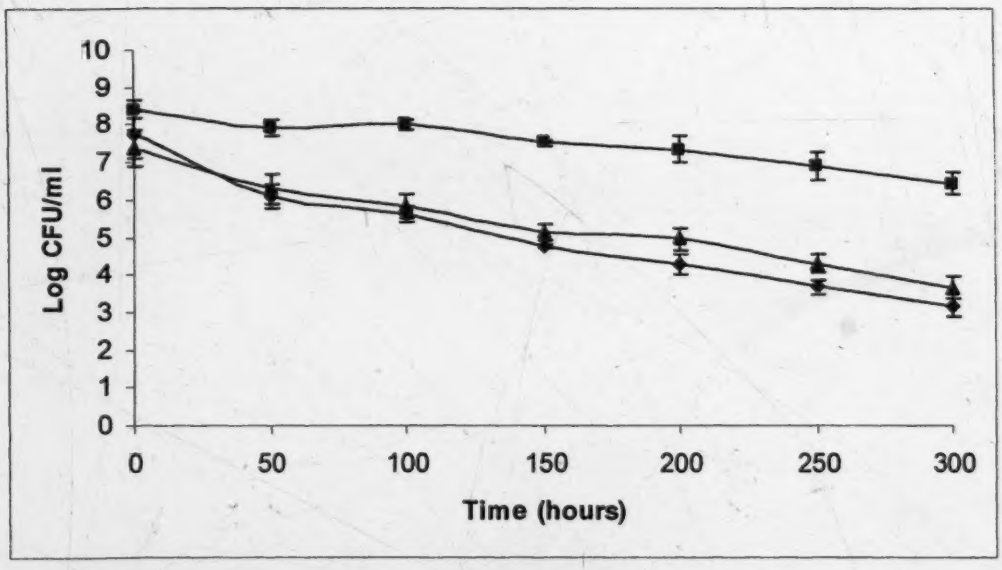

Figure 5. Salt tolerance of strains of *L. monocytogenes* ATCC 15313 wild-type (♦), Leu^r (■) and bro^r (▲) resistant strains in 20% NaCl

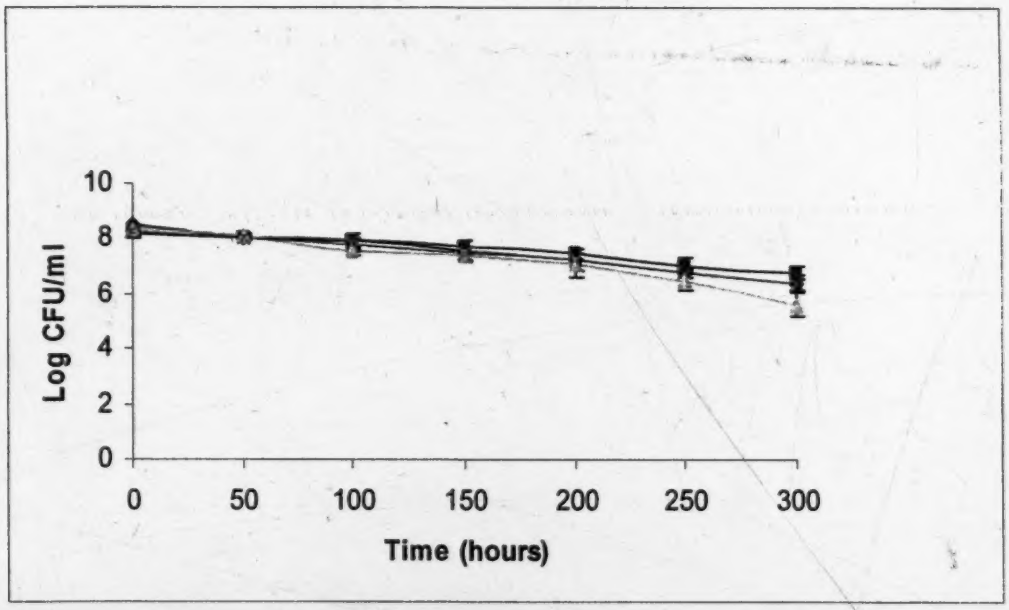

Figure 6. Death curves of *L. monocytogenes* Scott A in 20% NaCl. Wild-type (♦), Leu^r (■),bro^r (▲) and bro-nisin ^{r} (×) strains

4.8 Tolerance to low pH

Sensitivity of *L. monocytogenes* strains to low pH was evaluated in TSBYE with 0.1 M acetic acid added to give pH 3.6. Samples were collected at predetermined times and plated on TSBYE agar for enumeration. Statistical analysis was performed by ANOVA among bacteriocin-resistant isolates compared with wild-type strains and significant differences ($p<0.05$) were found between at least two of the samples that were included in the study.

4.8.1 *L. monocytogenes* ATCC 15313

Tukey's test revealed that strains of *L. monocytogenes* leu^r 15313 had a higher tolerance to pH ($p<0.05$) compared with the pH tolerance of the wild-type and bro^r strains,

however, the wild-type and 15313 bro^r strains were not significantly different ($p>0.05$) in their tolerance to pH 3.6 (Figure 7).

4.8.2 *L. monocytogenes* Scott A

Tukey's test revealed that wild-type and leu^r strains were not significantly different ($p>0.05$). In addition, bro^r and bro-nis^r strains were not significantly different ($p>0.05$). A significant difference between the wild-type strain and the two bro^r strains was found. This indicated that strains that were resistant to brochocin-C were more tolerant to low pH (3.6) than the wild-type (parental) strain (Figure 8).

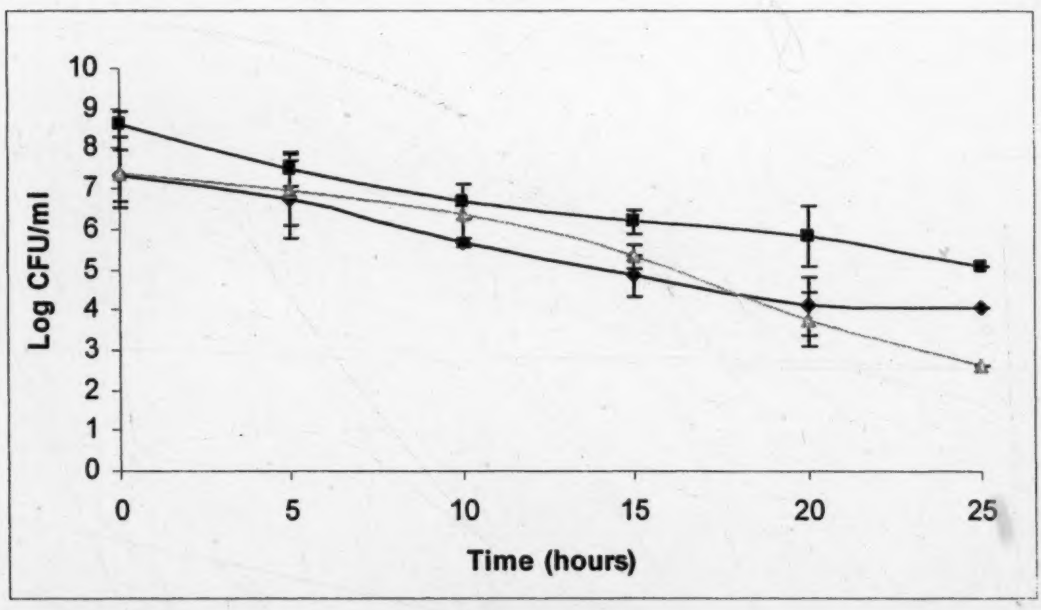

Figure 7. Death curves of *L. monocytogenes* ATCC 15313 strains in acetic acid 0.1 M pH 3.6. Wild-type (♦), Leu^r (■), bro^r (▲) strains

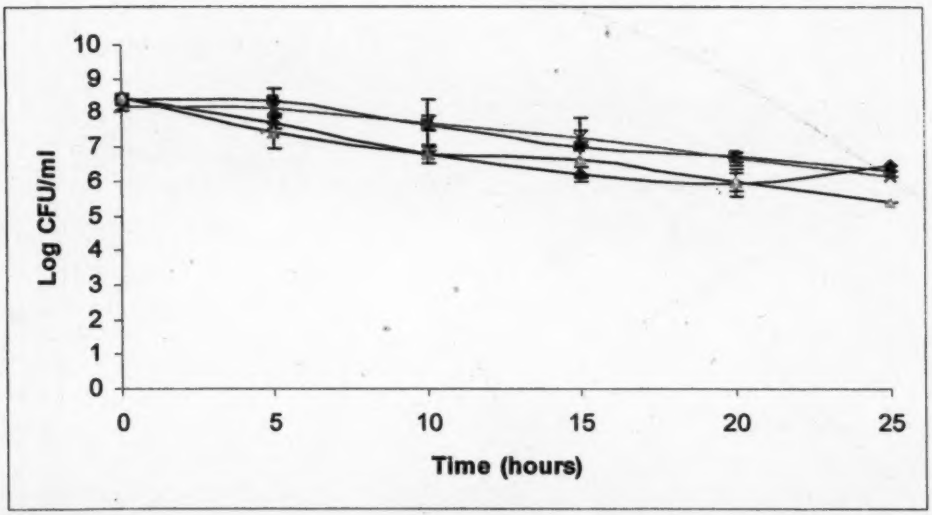

Figure 8. Death curves of *L. monocytogenes* Scott A strains in acetic acid 0.1 M pH 3.6. Wild-type (♦), Leu^r (▲), bro^r (■), bro-nisin^r strains (×)

Table 13. Summary of statistical significance difference ($p < 0.05$) of strains of *L. monocytogenes* resistant to bacteriocins and resistance to other characteristics.

Strains resistant to bacteriocins	Heat	Salt	Acid
15313 bro^r			
15313 leu^r			
Scott A bro^r			
Scott A bro-nis^r			
Scott A leu^r			

Statistical significant difference between strain resistant to bacteriocins and the wild-type

5 Discussion

L. monocytogenes is difficult to control in foods because of its tolerance of adverse environmental and preservation factors used in food systems such as high temperature, low pH, salt concentration and refrigeration. This resistance is remarkable because it affords this pathogenic organism the potential to survive the "hurdles" introduced by a range of mild preservation treatments such as mild heating, vacuum or modified atmosphere packaging and natural antimicrobial systems used to delay deterioration in the sensory and nutritional properties of a food. The different preservative hurdles used in food often have a synergistic effect but it has been demonstrated that sublethal exposure to stressors such as heat (Farber and Brown 1990; Linton *et al.* 1990); acid, starvation, ethanol, hydrogen peroxide (Lou and Yousef 1996); and osmotic shock (Jørgensen *et al.* 1995) can increase the thermotolerance of *L. monocytogenes.* Furthermore, stress adaptation to one of the stressors can confer resistance to other stressors thereby jeopardizing the safety of foods that are preserved with hurdle technology as well as foods that are processed by traditional technologies that use preservation treatments at the minimum levels required to ensure food safety and at the same time keep an acceptable sensory quality of the food.

Bacteriocins have been used as one component of the preservative factors that interact in hurdle technology in dry sausages (Campanini *et al.* 1993; Foegeding *et al.* 1992) and chicken sausages (Baccus-Taylor *et al.* 1993). Nisin has been used in combination with bacteriocinogenic lactic acid bacteria (Schillinger *et al.* 1998), the lactoperoxidase system

in milk (Boussouel *et al.* 1999) and with other preservatives such as lactic acid, NaCl and monolaurin (Bouttefroy *et al.* 2000; Parente *et al.* 1998; Thomas and Wimpenny 1996).

The occurrence of bacteriocin-resistant strains of foodborne pathogens that are normally sensitive to bacteriocins is a major concern for the use of bacteriocins as biopreservatives. Nisin resistance has been reported in *L. innocua* (Maisnier-Patin and Richard 1996) and *L. monocytogenes* (Bouttefroy and Millière 2000; De Martinis *et al.* 1997; Ferreira and Lund 1996; Mazzotta *et al.* 2000; Ming and Daschel 1993; Ukuku and Shelef 1997) and resistance to Class IIa bacteriocins has also been investigated (Dykes and Hastings 1998; Ennahar *et al.* 2000a; Gao 2001; Rasch and Knøchel, 1998; Rekhif *et al.* 1994; Vignolo *et al.* 2000). It has been suggested that bacteriocin resistance mechanisms could activate the stress response system triggering the synthesis of general stress proteins (Gao 2001) that may enhance the survival of bacteria to other preservation factors such as high temperature, low pH and elevated salt concentration. A similar phenomenon was reported in *L. monocytogenes* that had been adapted to survive acid conditions and conferred cross-protection to nisin (van Schaik *et al.* 1999).

Brochocin-C resistant *L. monocytogenes* Scott A and ATCC 15313 were detected in this study at frequencies of 10^{-3} with 100 AU/ml and 10^{-5} to 10^{-7} at higher concentrations of brochocin-C, which suggests that concentration of brochocin-C is a factor in development of resistance to this bacteriocin. Similar results were reported by Gao (2001) for *L. monocytogenes* ATCC 15313. The nisin resistance frequencies in strains of *L. monocytogenes* were found at frequencies of 10^{-4} and 10^{-6} in both strains used. After

exposure to nisin, both strains used in this study lost the resistance to nisin after two subcultures in the absence of nisin as a selective pressure. This contrasts with the stable nisin resistance reported by other researchers (Mazzotta and Montville 1997; Ming and Daschel 1993; Schillinger *et al.* 1998).

The frequency of leucocin A resistance was not determined in this study because the procedure used to obtain resistant cells did not allow quantification. The cells were exposed to leucocin A in TSBYE broth instead of agar as used for nisin and brochocin-C resistance.

The stability of the resistance character was studied in strains with a single exposure and with strains sequentially exposed with increasing concentrations of bacteriocins in 100 AU steps up to a final concentration of 500 AU of brochocin-C and leucocin A. Multiple exposure of the strains to bacteriocins developed stable bacteriocin resistance even after growth for one hundred generations in the absence of bacteriocin, whereas strains with a single exposure to bacteriocin gradually regained their bacteriocin sensitive characteristic. Similar results were reported by Gao (2001) and Rekhif *et al.* (1994). This suggests that the generation of bacteriocin-resistant strains could result from selection of a natural resistant subpopulation. It is possible that a subpopulation of bacteriocin-resistant cells is present in a *L. monocytogenes* culture and that the resistant cells "emerge" as the dominant population when they are grown in the presence of a bacteriocin to which the strain is sensitive. Alternatively, mutation of some cells could occur when organisms are exposed to bacteriocins and these cells survive as resistant strains. However, divergent results have also been reported. Dykes and Hastings (1998)

reported the occurrence of an unstable class II bacteriocin resistance character in *L. monocytogenes* strains after ten cycles of growth without bacteriocin. These discrepancies suggested that bacteriocin resistance is a complex phenomenon and different levels of resistance may be generated.

The development of resistance to one bacteriocin resulting in resistance to other bacteriocins (cross-resistance) has frequently been reported. (Dykes and Hastings 1998; Gravesen *et al.* 2002b; Rasch and Knøchel 1998; Rekhif *et al.* 1994; Song and Richard 1997). Similar results were observed in this study. Brochocin-C and leucocin A resistant strains showed cross-resistance between the different strains; however, they retained their sensitivity to nisin (class I bacteriocin) as previously reported (Bouttefroy and Millière 2000; Dykes and Hastings 1998; Gao 2001; Rasch and Knøchel 1998; Rekhif *et al.* 1994; Schillinger *et al.* 1998). This suggests that the mechanism of nisin resistance is different to the mechanism involved in resistance to class II bacteriocins.

Determination of the genetic profile of bacteriocin-resistant strains was performed to confirm the certainty of parental relationship between wild-type and bacteriocin resistant strains used in this study. RAPD analysis was chosen for this purpose because it is highly sensitive and it has been successfully used as a tool in differentiation of *L. monocytogenes* strains (Farber and Addison 1994; Fonnesbech *et al.* 2001; Mazurier and Wernars 1992; O'Donoghue *et al.* 1995). Based on the results, it was shown that the bacteriocin-resistant strains used in the lethal preservation experiments had the same RAPD profile as the wild-type (parental) strains.

Bacterial tolerance to heat is difficult to study because there are so many variables and conditions that affect thermal tolerance of a bacterial cell, such as heating menstrum, composition of the growth medium and previous growth conditions (Doyle *et al.* 2001). As a result it is difficult to compare the results of this study with those of previous studies. However, the main purpose of the thermal tolerance experiments was to compare heat-tolerance between bacteriocin-resistant strains and the wild-type (parental) strain under the same experimental conditions. Thermotolerance of bacteriocin-resistant strains of *L. monocytogenes* compared with their parental wild-type strains showed that the resistant *L. monocytogenes* Scott A and ATCC 15313 strains were not less heat-resistant than their parental strains. Strains of leucocin A resistant *L. monocytogenes* ATCC 15313 showed a significant increase in thermotolerance ($p<0.05$) in comparison to that of the wild-type and the brochocin-C resistant strains. This suggests that the acquisition of resistance to leucocin A may influence the sensitivity of the strain to heat and therefore may affect the efficacy of thermal process against *L. monocytogenes.*

The ability of *Listeria monocytogenes* to tolerate salt stress is of particular importance because this pathogen is often exposed to environments containing elevated salt concentrations during food processing and food preservation (Duche *et al.* 2002), The data for salt tolerance observed in this study showed some similarities to the data for thermotolerance in which the leucocin A resistant strain of *L. monocytogenes* ATCC 15313 had a significantly ($p<0.05$) higher salt tolerance compared with that of the wild-type and brochocin-C resistant strains. Although the brochocin-C resistant strain was not more salt tolerant than wild-type strain, it was not more sensitive. These data were in

accordance with results reported by Gravesen *et al.* (2002a) in which nisin and pediocin PA-1 resistant mutants were not more sensitive to low pH and elevated NaCl concentration. In contrast, Mazzotta *et al.* (2000) reported that nisin-resistant cells were more sensitive to NaCl, $NaNO_2$, potassium sorbate and low pH than the comparable wild-type *L. monocytogenes* cells.

To cause illness, *L. monocytogenes* must survive the acidic environment of the stomach before it reaches the intestine. Thus, the acidity of gastric juice provides a first line of defense against foodborne pathogens. When *L. monocytogenes* acquires increased acid tolerance as a result of acid shock or other stressors that trigger the general stress response system of the bacterial cell, the chance of the cell surviving and proliferating in acidic foods such as apple cider, mayonnaise, sausages, etc. is increased. Thus it may survive the passage through the stomach and the acidic environment in the phagolysosomes (Lou and Yousef 1997; O'Driscoll *et al.* 1996). The acid tolerance data showed that the leucocin A resistant strain of *L. monocytogenes* ATCC 15313 was significantly ($p<0.05$) more acid-tolerant than the wild-type strain. However, the brochocin-C resistant strain was not significantly ($p>0.05$) different in acid tolerance than the wild-type strain. This indicates a difference in the mechanism of resistance among the leucocin A and brochocin-C resistant strains and that in the case of leucocin resistance there is increased resistance to acidic conditions.

In the case of *L. monocytogenes* Scott A, strains that were resistant to brochocin-C and strains that were resistant to both nisin and brochocin-C showed a significant ($p<0.05$) increase in acid tolerance than the wild-type and also the leucocin A resistant strain.

Meanwhile, the leucocin A resistant strain did not differ in acid tolerance compared with the wild-type strain.

Acetic acid was chosen as the acidifying agent for this study because it is highly protonated (99.5%) at the pH level (3.6) studied, therefore, the acid molecules readily pass across the cytoplasmic membrane and dissociate inside the cell cytoplasm decreasing the internal pH of the cell. Moreover the lethal effect of acetic acid in *L. monocytogenes* is well documented (Greenacre *et al.* 2003; Ostling and Lindgren 1993; Young and Foegeding 1993).

To avoid the emergence of bacteriocin-resistant strains of *L. monocytogenes* after exposure to bacteriocins it may be necessary (i) to use multiple bacteriocins from different classes of bacteriocins, and (ii) to use bacteriocins in combination with other preservatives to enhance the lethal effect and therefore the safety of foods. However, as shown in this study, bacteriocin resistance can be a stable condition and the effectiveness of the use of multiple bacteriocins from the same class, i. e., Class II, could be diminished. Furthermore, the resistant strains could survive and grow as well as wild-type strains and in some cases they can develop increased resistance to lethal preservation factors as shown in this study for leucocin A resistant strains. This study confirms that acquisition of bacteriocin tolerance in strains of *L. monocytogenes* could alter their resistance to other preservation factors and have significant food-safety implications, especially in situations where the food industry explores low levels of lethality as in minimal processing technologies.

6 References

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